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ON QUANTITATIVE MUSCLE PROTEIN DETERMINATION

Sarcoplasm and Myofibril Protein Content of Normal and Atrophic Skeletal Muscles

By

EINAR HELANDER

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PREFACE

This investigation, commenced in 1949, was made possible above all by the interest and personal inspiration accorded me by Professor Bo Erik Ingelmark, M. D. He first directed my attention to the subject of muscular tissue adaptation under different normal and abnormal conditions. I am exceedingly grateful to him for years of patience and for the facilities he made available to me at the Institute of Anatomy of the University of Gothenburg.

Through the courtesy of Professor Arne Tiselius, Ph. D., M. D., I was given the opportunity of working for a long time at the Institute of Biochemistry of the University of Upsala. I am greatly indebted to him for his interest in my work and for the facilities put at my disposal.

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Gothenburg in March 1957.

Einar Helander.

INTRODUCTION

A muscle cell contains myofibrils surrounded by sarcoplasm (Fig. 1). Muscle cells are held together by connective tissue which contains nerves, vessels and a small amount of fat, all of which henceforth will be called the stroma.

The parts played by myofibrils and sarcoplasm have been much debated (KÜHNE, 1888; LEWIS, 1915; LEWIS & LEWIS, 1917; RYDÉN & WOHLFART, 1932; DE RENYI & HOGUE, 1939; BARER, 1948), but nowadays it is generally thought that the myofibrils alone are responsible for muscular contractility (BUCHTAL & LINHARD, 1939). Opinions differ as regards the changes that take place inside the muscle cell during contraction (BUCHTAL, KNAPPEIS & LINHARD, 1936; HUXLEY & HANSON, 1953, 1954, 1955; SJÖSTRAND & ANDERSSON, 1956).

The sarcoplasm seems to have an important function as a storehouse for muscle cell nutrients. Investigations performed during the last few decades have shown that it is the site of a number of enzymes which are essential to carbohydrate metabolism (ENGELHARDT & LJUBIMOWA, 1939; HOUSSAY 1956;). Hence, the sarcoplasm certainly would seem to take a more active part in the intermediate metabolism than had been thought previously.

Considering that the myofibrils are solely responsible for the contractility while the sarcoplasm plays a more passive part, the contractile power of the muscular cell would seem to be dependent on the fibril density. The fewer fibrils there are in a cell, the less its contractile power should be, and vice versa.

By various means, mostly morphological, (KNOLL, 1891; HEIDENHAIN, 1911) attempts have been made to estimate the amount of sarcoplasm and myofibrils in the normal muscle cell. According to HASSELBACH (1953), and BUCHTAL (1957) who studied muscles from rabbits and frogs respectively, about 60 per cent of the cell's volume is occupied by myofibrils.

Many papers discuss the changes in the proportions of sarcoplasm and myofibrils to be found in abnormal muscle cells. They tell us

that reduction and ultimately loss of contractility is accompanied by diminution and loss of the muscle cell's double refraction (ADAMS, DENNY-BROWN & PEARSON, 1953). Such alterations are seen in various muscular diseases and following experimental muscular atrophy, e. g. that induced by denervation. On the other hand, the effect of muscular excercise has been the subject of much speculation, and the strengthening of the muscles resulting therefrom has been attributed to augmented muscular volume and/or to increased fibril density.

However, only in exceptional cases do investigations such as those mentioned in the foregoing provide any information regarding the actual quantities of sarcoplasm and myofibrils in the muscle cells. In most cases, one finds merely rough qualitative estimates of changes in the proportions of these structures.

The aim of the investigation here presented has been to attempt quantitatively to determine the amounts of sarcoplasm and of myofibrils existing in muscular tissue taken from both normal and atrophic muscles. The work is in two parts. In the first part, the author has elaborated methods for quantitative determination of the sarcoplasm and myofibril contents. Since it is well known from numerous previous investigations which proteins are bound to sarcoplasm and to myofibrils respectively, a conception of the amounts of sarcoplasm and myofibrils present can be obtained by determining the amounts of such proteins. The author has consequently studied the prerequisites for using biochemical methods of extracting and quantitatively assessing the protein content of the muscle.

In the second part of the work, the author has used these techniques to determine the quantities of sarcoplasm protein and of myofibril protein in normal and atrophic muscles, the muscular atrophy being induced experimentally by tenotomy, neurotomy and inactivity due to applied plaster casts.

Before the experiments are described, it seems appropriate to provide a brief survey of the main characteristics of the muscle cell proteins, as well as a review of previous papers reporting attempts to determine these proteins quantitatively in normal and atrophic musculature.

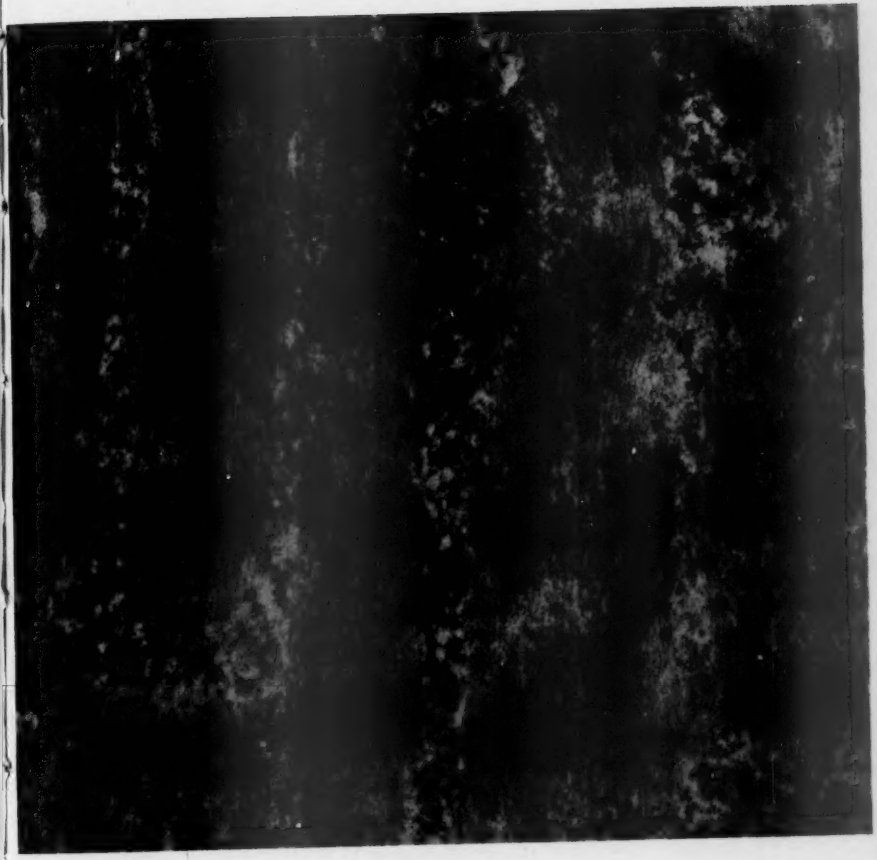

Fig. 1. Electron micrograph of part of a muscle cell. Magnification 28,500 x.

Chapter I

MUSCLE CELL PROTEINS

The muscle cell contains a large variety of proteins, and our knowledge about them is extensive. This applies particularly to those proteins that take part in the carbohydrate metabolism of the muscle cell and in the contraction process. Owing to its profusion, the relevant literature is most difficult to systematize, notwithstanding the existence of some survey papers (BAILEY, 1944; A. SZENT-GYÖRGYI, 1944; WEBER, 1950; DUBUISSON, 1954; PERRY, 1956). In the light of numerous investigations (WEBER, DUBUISSON, SZENT-GYÖRGYI), it must be taken as an established fact that the following proteins are bound to the sarcoplasm: myogen, myoalbumin, globulin X , and myoglobin. When these are extracted selectively, the myofibrils remain intact (PERRY, 1952).

The myofibril proteins are generally called contractile proteins. Myosin, actin and the compound of these two, actomyosin, belong to this category. So do tropomyosin and a number of recently discovered myosin-like proteins, e. g. extra protein (EP). However, little is yet known about these.

The function of actin and myosin in the muscle cell has been the subject of various opinions. Some recent electron microscopical investigations of myofibrils (HASSELBACH, 1954) have demonstrated that the A-bands become more translucent following selective extraction of myosin. After such extraction, only thin, lengthwise filaments resembling strings of pearls remain in the fibril. Because of their appearance, these filaments have been assumed to consist of actin. If so, it follows that myosin is present in the A-bands exclusively.

Previous studies on muscle proteins have dealt mainly with their qualitative attributes. The present author aims to discuss the possibility of recovering these proteins quantitatively. Hence, the main biochemical properties of these substances and the procedures formerly adopted for obtaining them will now be outlined briefly.

The first studies on muscle extracts were made as early as the middle of the 19th century by KÜHNE (1864, 1868, 1889), HALLIBURTON (1887 a, 1887 b) and DANILEWSKY (1882). Obviously, it would be carrying things too far to consider all the different kinds of proteins obtained and defined by biochemical techniques belonging to another era. The following particulars have, in the main, been confined to papers published during the past three decades — a period characterized by remarkable advances in our knowledge of muscle proteins.

A. *Sarcoplasm Proteins.*

1. *Myogen.* When a finely subdivided muscle is extracted with distilled water, about one third of its proteins will dissolve. The fraction thus obtained has been named myogen, and what remains has come to be known as myosin. The properties of myogen have been studied by WEBER and by BARANOWSKI who approached the problem along completely different lines.

WEBER prepared myogen by subjecting minced muscle to high pressure. The muscle juice thus expressed was filtered and dialyzed against distilled water. Myosin and globulin X were then precipitated, while myogen remained in solution. The specific gravity of a myogen solution is 1.35 (WEBER, 1927 a) and its viscosity is low (WEBER, 1925 a, 1934 a; WEBER & RONA, 1928). The isoelectric point of myogen lies at pH 6.3 (WEBER, 1925 a) or between 6.5 and 6.7 (BATE-SMITH, 1937). Its molecular weight as estimated by DEUTICKE is in the range 80,000—100,000 (1934); according to STÖVER it is about 81,000 (1933). The nitrogen content of myogen is stated to be 16.6 per cent (WEBER, 1934 a; BAILEY, 1937).

BARANOWSKI, on the other hand, obtained myogen in the following manner (1939 a, 1939 b): Muscles from exsanguinated rabbits were subdivided in a mincing machine, stirred with ice water and filtered. The filtrate was allowed to stand for six hours at room temperature before being centrifuged. The solution could be fractionated by addition of ammonium sulphate, myogen A crystallizing out mainly as hexagonal prisms and myogen B predominantely as long asymmetric plates.

The solution yielding myogens A and B contains a number of other proteins. Using a salting-out technique, DISTÈCHE (1948 a, 1948 b) found eight different proteins precipitating between 50

and 66 per cent saturation with ammonium sulphate, and he could crystallize five of them.

A myogen A solution has a low viscosity. The isoelectric point lies at pH 6.3. Its molecular weight, as determined by GRALÉN (1939), is about 150,000. Its nitrogen content is 15.52 per cent. The substance is believed to contain a series of enzyme fractions, for example aldolase, 1-α-glycerophosphatase, dehydrogenases and triose phosphate isomerase (MEYERHOF & BECK, 1944; BARANOWSKI, 1949). Myogen B has prethixotropic properties. Following saturation with ammonium sulphate, it shows double refraction of flow, but this phenomenon disappears entirely after dialysis. Little else seems to be known about myogen B.

2. *Myoalbumin.* This substance has been studied by BATE-SMITH (1937). It is obtained by extracting muscle with distilled water. A myoalbumin solution has low viscosity and lacks double refracting properties. The isoelectric point lies at pH 3—3.5 according to BATE-SMITH, and at 4.65 according to JACOB (1948). This discrepancy might be due to the use of different buffers and temperatures or to the fact that BATE-SMITH's myoalbumin was considerably modified and adsorbed on quartz particles.

3. *Globulin X.* WEBER was the first to prepare globulin X (1934 a) When dialyzed against water, the extract mentioned previously in connection with myogen yielded, at ionic strength μ[1]) 0.005, a precipitate of globulin X. The substance is slightly viscous in solution. Its isoelectric point is stated to lie at pH 5.0 (BATE-SMITH, 1937), and its estimated molecular weight is about 160,000 (WEBER, 1934 a) Like myoalbumin, globulin X has been little studied, and it probably is a mixture of different proteins (DUBUISSON, 1954).

4. *Myoglobin.* This, the first muscle protein ever studied, is obtained by extraction with water. The chemical properties of myoglobin have been established mainly as a result of THEORELL's (1932, 1934, 1947) researches. It is easily soluble, globular, and has a low molecular weight of the order of 17,000 (SVEDBERG, 1938). The isoelectric point lies at pH 7.0, and the absorbtion spectrum is characteristic.

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[1]) ionic strength calculated according to the formula $\mu = {}^1/_2 \Sigma c Z^2$.

In summary, it may be stated that sarcoplasm proteins have a number of physical and chemical properties in common. They are globular, have low viscosity, low molecular weight and are easy to extract.

B. *Myofibril Proteins*

1. *Myosin, Actin and Actomyosin.* Myosin was extracted and studied already in the 19th century. However, real advances in our knowledge of the substance came only in the 1920's, mainly due to the researches of WEBER and his school. (WEBER, 1925 a, 1925 b, 1927 a, 1927 b, 1934 a, 1934 b; WEBER & RONA, 1928; BOEHM, 1930; BOEHM & SIGNER, 1931; BOEHM & WEBER, 1932; MEYER & WEBER, 1933; STÖVER, 1933).

From muscle tissue, WEBER isolated a substance he called myosin which precipitated when the solution was diluted with distilled water. This protein sediment again dissolved when 0.5 M KCl was added at pH 7.4. The next investigator of myosin was EDSALL (EDSALL, 1930; v. MURALT & EDSALL, 1930 a, 1930 b, 1930 c; EDSALL et al., 1939; EDSALL & MEHL, 1940; GREENSTEIN & EDSALL, 1940), and he was followed by a long line of other researchers.

Through the work of A. SZENT-GYÖRGYI and his coworkers (Enzimologia 1940, Stud. Inst. Med. Chem. Univ. Szeged 1941—1943; A. SZENT-GYÖRGYI, 1944) it was established that the myosin produced by WEBER and EDSALL was composed of two fractions, now known as myosin and actomyosin, and, when STRAUB (1942, 1943 a) prepared actin, it was concluded that actomyosin consisted of actin bound to myosin.

a. *Myosin.* Myosin is prepared by extracting minced muscle with 0.3 M KCl + 0.15 M K phosphate at pH 6.5 for ten minutes at 0° C under constant stirring. Then the muscle tissue is removed by centrifuging and the supernatant diluted with 4 volumes of water at room temperature. After one to two hours a precipitate has appeared which consists of actomyosin. When this precipitate has been filtered off the solution is again diluted with 1.5 volumes of cold water and allowed to stand for 2 hours at 0° C. Myosin will now crystallize out as small needles (A. SZENT-GYÖRGYI, 1944). A myosin solution shows double refraction of flow and is viscous (GUBA & STRAUB, 1943). The isoelectric point in 0.28 M KCl solution

lies at pH 5.4, but appears to vary depending on the composition of the salt solution (HOLLWEDE & WEBER, 1938; ERDÖS & SNELLMAN, 1948; SNELLMAN & ERDÖS, 1948 a).

Different figures are given for myosin's molecular weight: SNELLMAN and coworkers (SNELLMAN & ERDÖS, 1948 b; SNELLMAN & TENOW, 1948) give 1,500,000 with which BERGGOLD concurred (1946), whereas PORTZEHL had about 850,000 (1950), PARRISH & MOMMAERTS 650,000 (1954) and LAKI & CARROLL 500,000 (1955). These differences must probably be ascribed to nonidentical experimental conditions (LAKI & CARROLL, 1955). Myosin contains 16.15 per cent of nitrogen. The action of adenosine-triphosphatase is associated with myosin.

According to PORTZEHL (1950), the length of the myosin molecule is 2,200—2,400 Å and its diameter 22—24 Å. SNELLMAN & ERDÖS (1948 c) have demonstrated that if weak salt solutions are used myosin looks like long thin threads, otherwise it forms a protein film.

b. *Actin.* This substance was discovered by STRAUB (1942, 1943 a) who prepared it by first extracting myosin and actomyosin from the muscle with a salt solution and then treating the residue with acetone. After dehydration of the remainder, actin can be obtained by extraction with water. This actin can be purified by MOMMAERT's technique (1951). Another procedure has been described by A. G. SZENT-GYÖRGYI (1951). Here the myosin is first removed by extraction in the usual manner, whereupon the extraction is repeated with the addition of small amounts of adenosine triphosphate. The muscle residue is then extracted with 0.6 M KI solution containing small amounts of sodium thiosulphate and adenosine triphosphate. Addition of an equal volume of water makes a sediment fall out which is removed before a small amount of alcohol is added. Another precipitate is now formed, and alcohol and KI can be removed from the final actin solution by dialysis.

Actin has been described by STRAUB and BALENOVIC (BALENOVIC & STRAUB, 1942; STRAUB, 1942, 1943 a). It occurs in two varieties: in a globular form known as G-actin, or polymerized to fibres, so-called F-actin.

G-actin is soluble in water, has a low viscosity and displays no double refracting properties. Its isoelectric point lies at pH 4.7 and the molecular weight is said to be about 80,000 (STEINER & LAKI,

1952), or according to others, 70,000 (TSAO & BAILEY, 1955). The nitrogen content is 15.1 per cent. Under the electron microscope, it has the appearance of small spherical particles.

F-actin is highly viscous, thixotropic and shows double refraction of flow. In the electron microscope, it appears as fibres and bundles. Certain observations suggest that these have the same length as the myosin particles. (ROZSA *et al*, 1949).

c. *Actomyosin*. The technique for extracting actomyosin is described above under myosin. The substance can also be produced by mixing F-actin and myosin. This process is reversible. New dissociation can be induced by such substances as adenosine triphosphate. Actomyosin solutions show strong double refraction of flow and are highly viscous. At pH 6.7 and ionic strength 0.28—0.27 they gel. Electron microscopical studies have revealed that this protein has a fibrous structure (JAKUS & HALL, 1947; ASTBURY, 1948 a; PERRY *et al*, 1948; SNELLMAN & ERDÖS, 1948 c). Some researchers have assumed that, in actomyosin, the actin and myosin constituents are combined in a stoichiometric relationship (SNELLMAN & ERDÖS, 1949; SNELLMAN, 1950; SPICER & GERGELY, 1951). Others (PORTZEHL *et al*. 1950) have the opinion that the formation of actomyosin can take place in different ways and that there exist several actomyosins with different sedimentation constants.

2. *Tropomyosin*. Tropomyosin was first described by BAILEY (1946, 1948). It is obtained by treating the muscle tissue initially with water, then with ethanol and finally with ether. The tissue is then dried and the tropomyosin extracted with 7 volumes of M KCl. In a saltless medium at pH 6.5—7.0, tropomyosin yields a viscous non-thixotropic solution which shows double refraction of flow. The isoelectric point lies at pH 5.1. The tropomyosin molecule is asymmetrical and weighs about 90,000 (BAILEY et al, 1948). The substance is unsoluble in water. With 0.5 M KCl, 20 per cent of it can be recovered from fresh muscle tissue and, with M KCl, 80 per cent. The degree of recovery does not increase with rising salt concentrations. Improved yield was BAILEY's aim in using ethanol dried muscle tissue. He assumed tropomyosin to be a prototype of myosin and suggested that it could be one of the units from which the myosin filament is elaborated. This is borne out by the fact

that tropomyosin and myosin have certain similar properties, e. g. the amino acid composition and the X-ray diffraction pattern According to BAILEY, tropomyosin is a part of the myofibril and its low solubility is due to strong electrostatic forces and perhaps also to association with lipids.

3. *Extra Protein.* SZENT-GYÖRGYI *et al.* (1955) showed that when free myofibrils were extracted, the solution contained excess protein in amounts up to 30 per cent of all protein recovered. This extra protein, or EP as it is also called, has been studied extensively by VILLAFRANCA (1956) who showed that it is not identical with either myosin or tropomyosin. It occurs in both polymerized and depolymerized forms. The molecular weight of the former type is 447,500 and that of the latter 155,000. Polymerized EP shows double refraction of flow and is highly viscous in low salt solutions. The viscosity declines if the salt concentration is increased and in 0.6 M KCl the intrinsic viscosity decreases to a limiting value of one tenth to one sixteenth of that in solutions of low salt content. Electron microscopical examination reveals that polymerized EP consists of fibres.

Thus the myofibril proteins in non-depolymerized states have several properties in common which differ considerably from these of the sarcoplasm proteins. Among such properties are the following: fibrous nature, high viscosity, high molecular weight and low solubility. The characteristic properties of sarcoplasm and myofibril proteins are as follows:

Sarcoplasm Proteins	*Myofibril Proteins*
Globular	Fibrous
Low viscosity	High viscosity
Low molecular weight (~ 80,000)	High molecular weight (~ 400,000—800,000)
Soluble in water or low salt solutions.	Insoluble in water and low salt solutions.

As appears from the preceding text, the protein constituents of sarcoplasm and myofibrils display marked differences as regards their physical-chemical properties. When, as in the present author's case, the object is to recover these proteins quantitatively, the

readily soluble sarcoplasm proteins should be fairly simple to deal with. The contractile proteins on the other hand can be expected to give more trouble due to their fibrous character and low solubility. This was confirmed when the author developed his own method for muscle protein extraction, and most of the experiments were accordingly designed to establish whether or not the fibre proteins could be recovered completely.

Chapter II

THE QUANTITATIVE PROTEIN COMPOSITION OF NORMAL AND ATROPHIC MUSCLES AS REFLECTED IN THE LITERATURE

How the proteins in muscular tissue are distributed quantitatively is a question that apparently has interested fairly few workers. Only such papers as have a direct bearing on the matter will be reviewed here, and the earliest investigations will not be discussed (e. g. HALLIBURTON, 1888; FÜRTH, 1893; SAXL, 1907; RITCHIE & HOGAN, 1929).

The most comprehensive investigations have been done by WEBER & MEYER (1933), BATE-SMITH (1934, 1937) and HASSELBACH & SCHNEIDER (1951). In addition, there are a number of less extensive studies where only one or a few of the several protein constituents have been determined quantitatively (cf. p. 19).

NOLL and WEBER (1935) stated that the total protein content of muscular tissue is 15 to 16 per cent, which is equivalent to 86 or 87 per cent of the dry substance.

MEYER and WEBER's investigation (1933) concerned the quantitative protein composition of skeletal muscles in rabbit. They obtained the proteins in the following manner. After the animals had been killed, their hind legs were perfused with Ringer's solution. The musculature was then minced and extracted with 0.6 M KCl. The extraction was repeated 5 times at pH 8.5—9 and subsequently 3—5 times more at pH 9.5. Thereby up to 85 per cent of the muscle's total nitrogen content was recovered. WEBER and MEYER analyzed both white and red rabbit musculature, the results being as shown below.

The myosin content was computed after sedimentation at pH 7 and ionic strength 0.04. The quantity of globulin X was obtained by separation at pH 5.4 in a 0.015 M KCl solution.

	Per Cent of Total Nitrogen in Muscle	
	White	Red
Nonprotein N	13	11
Myogen N	19	15
Globulin-X N	19	15
Myosin N	34	35
Non soluble (stroma) N	14	24

After the extraction, the authors stained the remaining muscle fibres according to VAN GIESON's method and examined them under the microscope. It appeared that here and there the fibres were still striated and doubly refracting. Regarding this as evidence that the myosin had been incompletely extracted from the muscle cells, the authors assumed that the myosin percentage they had obtained was too low. As HASSELBACH & SCHNEIDER (1951) also have pointed out, the content of contractile protein must surely have been calculated too low and the myogen proportion too high, owing to the extraction procedure used. At the high pH levels employed, actin is irreversibly transformed into G-actin whose solubility is similar to that of the myogen fractions.

In two papers, BATE-SMITH (1934, 1937) has discussed procedures for extracting muscle proteins and presented quantitative results. The author experimented with rabbits in which the thoracic aorta was perfused with 1 per cent NaCl solution. In his first investigation, the author ground down the tissues with sand and extracted with a 5 per cent solution of $MgSO_4$, the grinding and extraction being repeated 9 times in succession. By this means, the author recovered 70 per cent of the protein nitrogen in the muscular tissues. Of the remaining 30 per cent, half was assumed to consist of extracellular proteins (collagen and elastin) and half of undissolved intracellular proteins. In his second investigation, BATE-SMITH used similar exhaustive extraction with 7 per cent LiCl solution. This time he could recover 82.5 per cent of the intracellular proteins (corresponding to about 70 per cent of the total protein nitrogen) and showed that his extract contained the following constituents (in per cent of total intracellular protein): myosin 63, globulin X 9 and myogen, together with myoalbumin, 10 per cent.

Bate-Smith thereupon washed the muscle residue with water twice and extracted several times with 0.01 N HCl. Having analyzed this last extract, he stated that three quarters of the residual intracellular protein was globulin X and one quarter was myosin. He therefore corrected upwards the figures he had obtained at first so as to get 67—68 per cent myosin, 20—22 per cent globulin X and 10—12 per cent myogen and myoalbumin.

Bate-Smith's extraction method and particularly his correction of the figures obtained directly have been strongly criticized by Kamp (1941) and Hasselbach & Schneider (1951).

Hasselbach & Schneider (1951) used musculature from exsanguinated rabbits which was immersed in ice water and then minced. The muscle tissue was then extracted with 4 volumes of 0.6 M KCl containing pyrophosphate at pH 6.0—6.5 and 0° C. Extraction with this solvent was repeated 5 times in succession, the first time for 20 minutes, the second for 1 hour, and the remaining 3 times for 2 hours each. The musculature was then put through the mincing machine twice and thereupon extracted for 18 hours without interruption. The residue thus obtained was extracted with a 30 per cent urea solution. The authors described an alternative procedure involving extraction periods totalling more than 50 hours.

The quantity of dissolved nitrogen, in terms of which the specified protein percentages were expressed, was then calculated from the formula

$$»N_D = C\ddot{u}\ (V_E + V_R),»$$

where N_D = amount of dissolved N, $C\ddot{u}$ = N concentration in extract, V_E = volume of extract collected, V_R = volume of muscle residue.

The authors stated that this extraction procedure had enabled them to recover 83—85 per cent of the muscle's total nitrogen. Of the protein fractions, 38 per cent consisted of myosin, 13—15 per cent of actin, 28 per cent of globular protein, and 4 per cent of urea extracted protein.

The literature includes a series of smaller investigations in which only one or a few of the constituents have been studied quantitatively. Balenovic & Straub (1942) extracted actin and stated that it constitutes 12—15 per cent of the muscle proteins. A. Szent-Györgyi (1944) reported that fresh muscle tissue contains 3 per

cent of actin and 8 per cent of myosin but only one third of this quantity was extractable by the KCl-phosphate solution. In another paper A. G. SZENT-GYÖRGYI and coworkers (1955) reported that myosin represents 25—30 per cent of the total muscle proteins.

Sarcoplasm protein determinations have been made by a number of investigators. They used either, but very seldomly, water or, more commonly, buffers of ionic strength about 0.08 as the extractant (DUBUISSON, 1950; PERRY, 1952). This extraction technique permits quantitative determination of the sarcoplasm proteins, because they all dissolve. This technique has also been employed for isolation of myofibrils.

Using electrophoretic methods, JACOB (1947 b) estimated the proportions of those proteins which dissolved at μ 0.05, i. e. the sarcoplasm proteins. It appeared that myoalbumin amounted to 8.5 per cent, phosphorylases to 13 per cent, myogen A to 24 per cent and the rest of the myogen fraction to 47.5 per cent. Some of the smaller fractions could not be reliably identified.

The occurrence of myoglobin in muscular tissue in man under various physiological and pathological conditions has been studied by BIÖRCK (1949). According to his findings, the myoglobin content of normal skeletal muscle is about 2.5 per cent of the dry weight.

Additionally, several investigators have studied the protein constituents in muscles from a variety of lower animals, e. g. haddock (REAY & KUCHEL, 1936), cod (DYER, FRENCH & SNOW, 1950) and torpedo (BAILEY, 1939).

No systematic investigation appears to have been carried out in which different methods were used to extract the greatest possible amount of the protein constituents from striated muscular tissue. The main investigations published so far are susceptible to criticism. As noted above, WEBER & MEYER's and BATE-SMITH's studies have already been questioned, and the objection may be raised against HASSELBACH & SCHNEIDER's findings that their method of computing the amount of dissolved protein rather than the amount extracted would seem to introduce a source of error, the effect of which upon the authors' quantitative estimations cannot be definitely evaluated for lack of complete data.

All these previous papers also have certain weaknesses in common. It has not been possible to extract more than about 80—85 per

cent of the total muscle proteins, so that as much as 15—20 per cent has been left over — an amount that would appear excessive if the residue consisted of stroma exclusively. Moreover, the extraction procedures employed have been far too complicated for general adoption. As a result, there have been no real opportunities for a comprehensive study of the quantitative distribution of the proteins in muscular tissue under different physiological and pathological conditions.

TABLE 1. *Quantitative protein composition of normal muscles according to various authors*

	Sarcoplasm Proteins	Myofibril Proteins	Stroma (including undissolved proteins)
WEBER & MEYER			
White Muscle	38	34	15
Red Muscle	30	35	24
BATE-SMITH			
Direct Figure	19	63	30
Corrected Figure	32	68	15
HASSELBACH & SCHNEIDER	28	52 (56)	17—15

A few papers discuss the protein composition of atrophic skeletal muscle. Considering that their results will be examined elsewhere (p. 82), a brief review of the methods adopted to determine the protein amounts will suffice at this point.

The simplest procedure used was to determine the total nitrogen content in the piece of muscle and then calculate directly the proportion of proteins from the result (CHOR, DOLKART & DAVENPORT, 1937; HUMOLLER, GRISWOLD & MCINTYRE, 1950).

Determinations of proteins have also been made using extraction procedures (CAHN, 1927; HINES & KNOWLTON, 1933; WESTENBRINK & KRABBE, 1936; FISCHER & RAMSEY, 1946 a). In most of these investigations now obsolete methods have been used to prepare the muscle specimen and extract the proteins. FISCHER & RAMSEY (1946 a), utilizing BATE-SMITH's technique (1937) described in the foregoing, reported the highest degree of recovery.

PART ONE

PROCEDURES FOR EXHAUSTIVE MUSCLE PROTEIN EXTRACTION

CAPTER III

MATERIALS AND GENERAL METHODS

1. *Materials*

The test animals used in all the experiments were white rabbits and weighed 1—2 kg. They were given an adequate amount of normal fodder. No animal exhibited any signs of disease, and no abnormalities of the internal organs were observed in any of those — about one third of the lot — which were examined *post mortem*.

The experiments were done with white muscle from the thigh. Whenever much tissue was required, muscles were excised from both thighs; otherwise only muscles from the right side were used. Then, in the manner detailed below, the muscles were rid of fasciae, fatty tissue, nerves, vessels and red muscle. If direct comparisons were considered necessary, e. g. when different preparation techniques were evaluated, the available excised muscle was divided up into portions that could be regarded as representative of the whole. When different extraction procedures were applied to frozen sections, the whole piece of muscle was first cut into sections whereupon these were mixed and then divided up into representative portions.

2. *General Methods*

The extraction solutions were prepared from analytical grade reagents and distilled water whose conductivity after treatment with ion exchangers was 0.7×10^{-6} mho/cm. The extracts were subjected to continuous slow agitation in a test tube rack. Extraction was performed in »Pyrex» glass tubes with precisely fitting stoppers.

Weights were determined with a »Sartorius» analytical balance having an accuracy of $\pm$ 0.1 mg. The weighing technique is discussed at length in a separate chapter (cf. p. 35).

The thermostatically controlled refrigerated room was held a temperature of $+2°$ C $\pm 1°$ C; room temperature fluctuated between

+18° C and +21° C; and the temperature in the deep-freezer was −20° C.

Dry weight determinations were carried out with the aid of a vacuum pump yielding a pressure of 10^{-2} — 10^{-3} mm Hg. Evacuation was continued until the same weight had been recorded three times in succession (two hours' interval).

Nitrogen determinations were made essentially according to JACOBS' (1951) modification of PREGL-PARNAS-WAGNER's (PARNAS & WAGNER, 1921) semi-micro technique. In this method, nitrogen is converted to ammonia and fixed as ammonium sulphate by 6 hours' digestion with concentrated sulphuric acid. Potassium sulphate is used to raise the temperature of the digestion mixture and copper sulphate as the catalyst. Thirty per cent hydrogen peroxide is added as the oxidant. The ammonia formed was liberated by addition of sodium hydroxide solution and was steam-distilled for 5 minutes, trapped in standard hydrochloric acid solution, and the excess hydrochloric acid was estimated titrimetrically with standard sodium hydroxide solution using methyl red as indicator. Non protein nitrogen was determined as the total nitrogen in extracts rendered protein free by precipitation with an equal volume of 20 per cent trichloroacetic acid (cf. HUXLEY & HANSON, 1957).

All the analytical results are derived from duplicate determinations. For calculating the protein percentages, the average nitrogen content of muscle proteins was taken as 15.5 per cent (BARANOWSKI, 1939 a, b; STRAUB, 1942, 1943 a; A. SZENT-GYÖRGYI, 1944).

The experimental results are uniformly stated in terms of mg's of nitrogen extracted from 1 g of muscle tissue and, unless otherwise noted, are based on at least 3, and usually 5 separate preparations. The amount of tissue in each sample generally ranged from 0.9 to 1.1 g.

The extracts were centrifuged with a refrigerated »Corda» machine producing a centrifugal force of about 1,400 g. Centrifuging was continued for 20 minutes. Rapid centrifuging was done in an »ICE» machine producing about 25,000 g, at a temperature of +2° C.

Viscosity measurements were made at +25° C in an »Ostwald» viscometer having a flow time of about 60 seconds for 2 ml of water.

pH determinations were made with an electric pH-meter (»Radiometer», type 23) having glass and calomel electrodes (input resistance: 10^{12} Ω).

Electrophoretic analysis was performed with the Tiselius apparatus (manufactured by LKB, type 3021) equipped with a Philpot-Svensson optical system (TISELIUS, 1937, 1938; PHILPOT, 1938; SVENSSON, 1939, 1940).

For subdividing the tissues, an all-glass Potter-Elvehjem homogenizer was employed.

Ultrasonic treatment was applied with a unit having a magnetostrictive oscillator and operating on a fixed frequency of 30 Kc/s. The total output power of the oscillator was estimated to 400 Watts (INGELMARK, 1946).

P^{32}-labelling of blood corpuscles was accomplished as outlined by HEDLUND (1953). The resultant radioactivity was detected and recorded with the same apparatus and technique as used by EKHOLM (1951).

For phase contrast microscopy a »Leitz» microscope was used (with magnification 8 × 20, numeric aperture 0.45).

Specimens were prepared for electron microscopy (Fig. 1) as follows. Excision was followed immediately by fixation in 1 per cent OsO_4 solution buffered with veronal acetate to pH 7.2 (PALLADE, 1952). The fixation solution was rendered blood isotonic by addition of NaCl solution. After dehydration the specimens were embedded in methacrylate, on the whole according to NEWMAN, BORYSKO & SWERDLOW (1949). Sectioning was done with a microtome of EKHOLM & ZELANDER's (1956) design and the micrographs were taken in an RCA EMU-3 b electron microscope.

Statistical analysis was performed according to DAHLBERG (1953).

CHAPTER IV.

EXPERIMENTS WITH PREPARATION TECHNIQUES

1. Killing the Animal and Estimating the Blood Quantity Remaining After Exsanguination

Introduction. The extant literature on muscle proteins is very uninformative as regards methods of devitalizing test animals and exsanguination procedures. EDSALL & GREENSTEIN (1940) reported that they »killed the animals by exsanguination under anesthesia». BARANOWSKI (1939) reported using exsanguinated animals. WEBER (1933) irrigated the large vessels with saccharose-saturated Ringer's solution in order to reduce oedema. BATE-SMITH (1934) perfused the aorta with 500 ml of 1 per cent NaCl solution. AMBERSON *et al.* (1949) stated that they exsanguinated the animals via the carotid artery while oxygenated Ringer-Locke solution was infused at the same rate through a cannula placed in the jugular vein. Hereby it was supposed that 96—98 per cent of the blood would be removed from the muscles. BIÖRCK (1949) determined the haemoglobin percentage in the dry substance of different muscles, finding that abdominal muscle contains 0.55 ± 0.093 per cent and extremital muscle 0.27 ± 0.034 per cent of haemoglobin. Both muscle categories were obtained at autopsy. The corresponding figures for the blood content per g of fresh muscle tissue would be about 0.9 and 0.4 per cent approximately (assuming that the water and haemoglobin contents of muscle are respectively 75 and 16 per cent).

The author's experiments. The animal was rendered unconscious by a sharp blow against the skull. Then the large cervical vessels were immediately severed and the blood allowed to run off while the animal was hung up in its lower extremities and vigorous thoracic massage was administered. When the animal is killed in this manner its extremital muscles perform repeated sharp contractions. All animals were killed at room temperature.

This clearly does not result in total exsanguination, so the amount of blood remaining must be determined. This could be accomplished with the aid of P^{32}-labelled blood corpuscles. Such blood corpuscles were injected intravenously 10 minutes before the animal was killed. The radioactivity was subsequently determined in venous blood and also in two extremely thin slices of thigh muscle that could be considered representative of the tissues to be used. When the muscles were being excised it was observed that they appeared absolutely bloodless and that blood occurred only in a few rather large extrafascicular vessels.

Results. Five animals were examined and from one of the corresponding thighs of these two pieces of muscle were taken for determination of the blood content. The results will be found in Table 2. As appears from the table the average blood content was 1.1 per cent.

TABLE 2. *Percentages remaining blood in exsanguinated muscles.*

Animal No.	Amount of blood in per cent of fresh muscle weight	
1	0.8	0.9
2	1.2	1.3
3	1.0	0.8
4	1.5	1.3
5	0.9	1.0
Average	1.1	

Discussion. In this investigation the method of killing the animals was designed to minimize blood contamination of the muscular tissue. This precludes the use of anaesthesia (e. g. with ether) to devitalize the animals, because, as shown by BIÖRCK and others, a much higher proportion of blood would then be present in the tissues. The reason for this is probably that the extremital muscles of the animal killed by anaesthesia fails to perform such vigorous contractions and therefore are less effectively emptied of blood. The amount of blood remaining — about 1 per cent — is so small that in my view it can be neglected. A blood content of 1 per cent implies that the proteins extracted from 1 g of muscular tissue will include about 0.002 g of blood proteins (haemoglobin + serum proteins). Under such

circumstances it seems unnecessary to supplement the exsanguination with other measures such as perfusion or compression. Such manipulations would on the contrary tend to delay the excision and freezing of the muscles. The latter would, as will be shown in due course, reduce the extractability of the fibre proteins.

2. Excision and Subdivision of the Muscle

Introduction. Previous authors have either subdivided the muscles more or less finely or, exceptionally, used them as they were (Amberson *et al.* 1949). Frequently the tissues have simply been cut up with scissors (Deuticke, 1930). A. Szent-Györgyi and his school generally put the muscle pieces through an ordinary chilled mincing machine with a front disc having 2 mm perforations (1944). The use of a machine having finer holes (such as Latapie's with 1 mm holes) failed to improve the extraction. Straub (1942, 1943 a), for his actin preparations, first ground the muscle in a cooled meat chopper and then in a cold Latapie mincer. Homogenizers have also been tried (Huxley & Hanson, 1957). Westenbrink & Krabbe (1936) froze the muscular tissue in liquid air, transferred it to dry ice, pulverized it with a hammer and passed it through a copper wire mesh to remove the larger fragments. Dubuisson and his school made use of a variety of preparation and extraction techniques and should therefore have considerable experience of the problem. Dubuisson thus stated in one of his publications (1950): »The coarsest method at our disposal is to pass the tissue through a meat-mincer (Latapie type), the finest to grind with fine sand the tissue previously passed through the mincer. The second technique might be expected to be more successful and, indeed, by this means cytologists such as Claude (1946 a, 1946 b)[1]) have been able to separate and isolate nuclei and even coramin threads. Between these two extremes lies a third rather less drastic method: the tissue is frozen solid, and sections 20—30 μ thick are cut by a freezing microtome. By this process it is possible to bring into solution a greater portion of some muscle proteins which are otherwise not easily accessible.

[1]) I have perused Claude's original publications without being able to verify these statements of Dubuisson's.

This method also has the advantage that the tissue is broken up, while the enzyme reactions are arrested by the low temperature (−5° C)».

The author's experiments. The muscle was excised at room temperature whereupon fasciae, intermuscular fatty tissue and visible nerves and vessels immediately were removed. This process takes between 30 and 60 seconds. Then the muscular tissue was subdivided by each of the methods to be described.

a. Without preparing the muscle in any way a die-shaped piece was simply cut out with a pair of scissors. This was weighed and extracted.

b. A large piece of muscle was put through a mincing machine placed in a refrigerated room. The product obtained was a mixture of solid muscle particles and juice. After this mixture had been stirred thoroughly a convenient amount was weighed and extracted.

c. In a refrigerated room the muscle was cut into as small pieces as possible with scissors, the resulting tissue pieces being about half the size of a grain of rice. These pieces were then extracted.

d. Muscle treated as in c was ground with sand in a mortar until (after about 5 minutes) a homogeneous product was obtained. This treatment took place in a refrigerated room and with a small quantity of extraction fluid added to the tissue. The sand proved impossible to remove so its presence was accepted during the extraction.

e. Homogenized muscle. A compact piece of muscle was weighed and reduced to portions of more convenient size with a few scissor cuts. These lumps were then homogenized for short periods for about 10 minutes altogether. Unavoidably a few muscle shreds remained unhomogenized. Moreover this procedure was attended by some heating of the tissue even though cooling periods were interposed. The homogenization was carried out in a refrigerated room and with extraction solution added.

f. Muscular tissue sectioned with the freezing microtome. In this case the freshly excised piece of tissue was frozen in carbon dioxide snow until a solid block had been formed which was affixed to the microtome table with a filter paper moistened in redistilled water. The tissue was then sectioned across the fibres into slices about 20 μ thick. A 3 or 4 mm thick slice of tissue adjacent to the microtome table and a similar slice facing the knife were discarded.

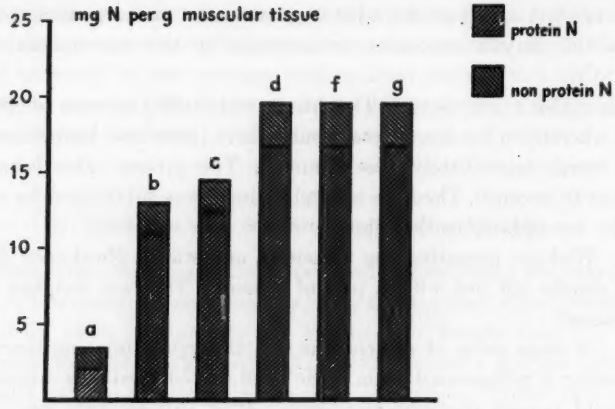

Fig. 2. Nitrogen yield using 0.6 M KCl after subdivision by various techniques.

a = compact block
b = minced
c = cut with scissors
d = as c and ground with sand
f = sectioned with freezing microtome
g = as f and ultrasonically treated

Sectioning was performed at room temperature. Before they had thawed the sections were put into a beaker containing carbon dioxide snow. Every precaution was taken to avoid heating the sections. Thawed sections could readily be distinguished because their colour and consistency were different. The sections were now weighed in the manner described elsewhere (cf. p. 35).

g. Muscular tissue treated as under f but immediately after addition of the extraction fluid subjected to ultrasound treatment in a test tube placed in the water above the oscillating surface for 20 minutes altogether. Owing to the nature of this treatment it was impossible to avoid a brief period of moderate elevation of the temperature which occasionally rose from + 2° to + 8° C. Whenever the latter temperature was attained the ultrasonic treatment was discontinued and the tissue cooled to + 2° C before fragmentation was resumed.

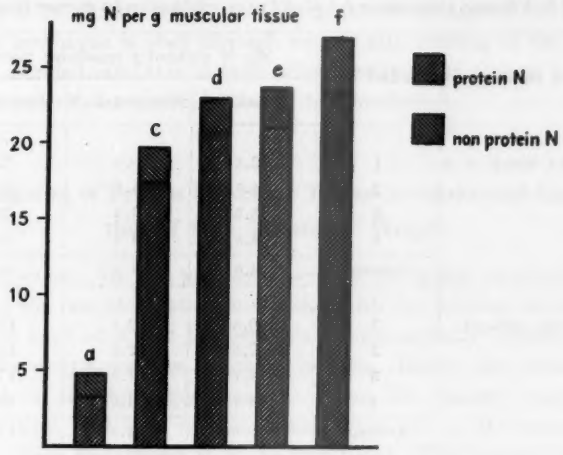

Fig. 3. Nitrogen yield using 1.1 M KI after subdivision by various techniques.

a = compact block
c = cut with scissors
d = as c and ground with sand
e = homogenized
f = sectioned with freezing microtome

Subdivision, however accomplished, was followed by extraction with two solutions. In one series extraction for 12 hours was performed in a refrigerated room with 10 aliquots of 0.6 M KCl + 0.05 M K phosphate buffer at pH 7.4 (the selected extraction fluid being considered representative of previous practice). This extraction procedure was adopted following subdivision by methods a, b, c, d, f, and g. The second series was extracted in a refrigerated room for 4 hours with 10 aliquots of 1.1 M KI + 0.1 M K phosphate buffer at pH 7.4. This was applied after subdivision as described under a, c, d, e, and f.

Results. Both extraction series comprised 4 experiments. Total nitrogen and non protein nitrogen were determined. The results of all series were mutually compatible. Averaged findings for each series are presented in Figs. 2 and 3 and Table 3.

TABLE 3. *Nitrogen yield using 1.1 M KI after subdivision by various techniques.*

Type of subdivision	Animal No.	Mg N yielded/g muscular tissue		
		Total-N	Nonprotein N	Protein N
Compact block = a	1	3.8	0.8	3.0
	2	4.7	1.5	3.2
	3	5.2	1.3	3.9
	4	4.8	1.3	3.5
	Average	4.6	1.2	3.4
Cut with scissors = c	1	20.0	2.3	17.7
	2	17.5	2.0	15.5
	3	19.1	1.9	17.2
	4	21.7	1.9	19.8
	Average	19.6	2.0	17.6
Cut with scissors and ground with sand = d	1	23.5	2.3	21.2
	2	21.1	2.8	18.3
	3	22.6	2.8	19.8
	4	23.0	2.4	20.6
	Average	22.6	2.6	20.0
Homogenized = e	1	23.1	2.9	20.2
	2	22.1	2.7	19.4
	3	22.7	3.1	19.6
	4	23.7	3.2	20.5
	Average	22.9	3.0	19.9
Sectioned with freezing microtome = f	1	25.9	3.3	22.6
	2	26.8	3.4	23.4
	3	26.6	3.4	23.2
	4	27.1	3.6	23.5
	Average	26.6	3.4	23.2

The experiments revealed that the most successful reduction was accomplished by cutting frozen sections.

Discussion. Most of the subdivision procedures adopted by the majority of previous authors have proved very inefficacious so far as quantitative recovery of muscle proteins is concerned.

As will be shown below, another great advantage of the frozen section technique is that through its use any heating of the tissue can be avoided, and this in turn solves the problem of how to store excised muscle, for example during transportation.

3. Weighing of Frozen Muscular Tissue Sections and Estimation of Dry Substance Weight

Introduction. In the preceding section particular emphasis was given to the fact that tissue subdivided with the freezing microtome could be kept at a low temperature uninterruptedly. Clearly this tends to complicate the weighing process. Hence the tissue was weighed in the following manner. From the beaker containing sections kept cold with carbon dioxide snow (cf. p. 31) convenient portions were transferred to extraction tubes. The nominal weight of the sections transferred to each such tube was 1 g, and this figure could be approached fairly closely after a little experience. When the sections were placed in the extraction tube — which was at room temperature — any adhering carbon dioxide snow rapidly evaporated. But a short time elapsed before the cold sections visibly began to thaw and meanwhile the extraction tube and its contents were weighed, with the stopper of the tube loosely inserted. Exceptionally, unevaporated carbon dioxide snow remained in the tube, a fact unmistakably manifested by the slowly declining weight of the tube, and in such cases this carbon dioxide snow was at once removed.

Although all the weighings were done in a uniform manner by the same person who took every reasonable precaution to guard against the presence of unevaporated carbon dioxide snow, a consistent error of method could affect the results because a minute amount of undetected carbon dioxide snow might have remained in the extraction tubes every precaution notwithstanding. Condensation of water vapour is another erratic factor that must be kept in mind. The following experiments were carried out in order to establish whether these sources of error had affected the results.

The author's experiments. Immediately after an animal had been killed and exsanguinated muscular tissue was excised from one

of its thighs. Seventeen pieces from this tissue were placed in weighing vessels having precisely fitting covers. The remainder of the muscle was frozen in carbon dioxide snow until a solid block was formed. This was sectioned with the freezing microtome. The sections were mixed thoroughly and, in the manner described above, 18 representative samples were transferred to extraction tubes which were weighed.

Then the amount of dry substance was determined by simultaneously evacuating all weighing vessels and extraction tubes to a pressure of 10^{-2} mm Hg. The samples were then weighed repeatedly until the weight was constant three times in succession and the water content could be calculated.

Results. The proportion of dry substance was 24.84 ± 0.246 per cent (range of variation 23.2—26.5) in samples taken directly from the muscle and 24.43 ± 0.098 per cent (range of variation 23.8 — 25.1) in the frozen sections. Thus the means for these two series did not differ significantly, although the range of variation was greater in the former.

Discussion. It appears from this experiment that carbon dioxide snow cannot have remained and water vapour cannot have condensed in the tubes. The statistically greater range of variation in the series of samples taken directly from the tissue must probably be attributed to non-uniformity of the samples themselves. Not so with the frozen section series, here sections representative of the entire muscle were mixed thoroughly so that each separate sample would be characteristic.

The following experiments included dry weight determinations on the muscle samples. As the dry weight only varied very slightly I deemed it satisfactory in connection with the several extraction experiments to express the proportion of nitrogen recovered in terms of milligrammes of nitrogen per gramme of fresh muscular tissue.

4. Muscular Tissue Storage at Different Temperatures Before and After Sectioning

Introduction. Most authors seem to have excised the muscle at room temperature, but full information on the subject is not seldom lacking. Some investigators have used liquid air as the refrigerant

(DEUTICKE, 1930; WESTENBRINK & KRABBE, 1936; CREPAX & HERION, 1954). PERRY (1952) stated that the muscles were quickly excised, iced and minced. SNELLMAN & TENOW (1948) froze the tissue with dry ice. A. SZENT-GYÖRGYI (1944) killed the animal, quickly skinned and eviscerated it and dipped it into ice-water. After some minutes the muscles were dissected out and chilled with ice. The latter procedure appears to be very common.

The fact that most investigators have subdivided the tissue by means other than cutting frozen sections has introduced an unavoidable delay between the excision and the chilling of the tissue. The excision process itself has also taken place at a variety of temperatures. Some have used a refrigerated room (AMBERSON *et al.* 1949; DUBUISSON, 1950) or added ice (A. SZENT-GYÖRGYI, 1944), others have worked at room temperature, again others have used various temperatures at different phases of the experiment.

The author's experiments. A. The aim of these experiments was to evaluate the influence of temperature on protein recovery from tissues stored at different temperatures before being subdivided. The following groups of comparative experiments were made.

a. The samples were kept continously deep frozen in carbon dioxide snow.

b. The pieces of tissue were placed in ice water for 30 minutes. In case this should have extracted some of the proteins the peripheral portions of the samples were removed before further treatment.

c. The samples were enclosed in a sealed tube for 60 minutes at room temperature.

d. The samples were enclosed in a sealed tube for 120 minutes at room temperature.

e. The samples were enclosed in a sealed tube for 60 minutes at a temperature of + 8° C.

f. The samples were inclosed in a sealed tube for 120 minutes at a temperature of + 8° C.

All the samples next were frozen simultaneously in carbon dioxide snow and sectioned with the freezing microtome. The samples were then weighed and extracted for 12 hours in a refrigerated room with 10 aliquots of 1.1 M KCl + 0.1 M K phosphate buffer pH

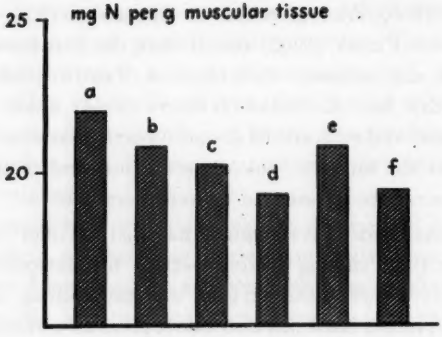

Fig. 4. Nitrogen yield using 1.1 M KCl after storage of muscle at different temperatures before freeze-sectioning.
Storage temperatures

a = −75° C
b = ice water
c = + 20° C, 60 mins.
d = + 20° C, 120 mins.
e = + 8° C, 60 mins.
f = + 8° C, 120 mins.

7.4 in one series and with 10 aliquots of 1.1 M KI + 0.1 M K phosphate buffer pH 7.4 in a second series.

B. Another lot of experiments was aimed to ascertain the influence of temperature on the protein recovery from tissue stored at different temperatures after sectioning in the frozen state. The following groups of comparative experiments were carried out.

The muscle as a whole was frozen solid in carbon dioxide snow and then sectioned. Convenient and representative samples of sections were weighed and transferred to extraction tubes which were sealed. These tubes then stored as specified under *a* and *c* to *f* above. Thereafter the samples were extracted for 12 hours in a refrigerated room with 10 aliquots of 1.1 M KI + 0.1 M K phosphate buffer pH 7.4.

C. A third set of tests was designed to establish how long frozen tissue sections can be stored in a deep freezer without adversely affecting the amount of proteins extracted. Frozen sections pre-

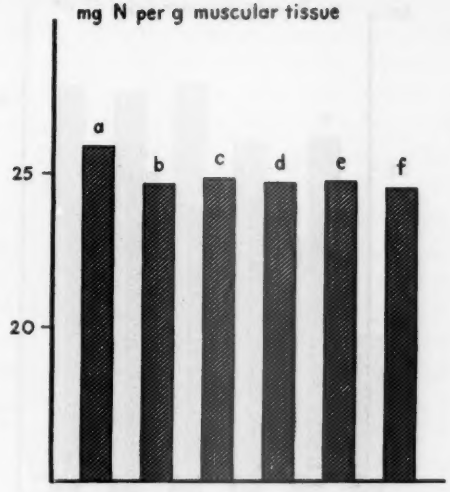

Fig. 5. Nitrogen yield using 1.1 M KI after storage of muscle at different temperatures before freeze-sectioning.
Storage temperatures

a = −75° C
b = ice water
c = + 20° C, 60 mins.
d = + 20° C, 120 mins.
e = + 8° C, 60 mins.
f = + 8° C, 120 mins.

pared from fresh muscle were put into tubes. These were sealed and stored in a deep freezer for a varying number of days before extraction commenced. The experiment was discontinued after 16 days. Extraction in a refrigerated room with 10 aliquots of 1.1 M KI + 0.1 M K phosphate buffer pH 7.4 was repeated thrice and lasted for 3, 3 and 2 hours respectively.

Results. The results of the first series of experiments (*A*) are presented in Figs. 4 and 5, Fig. 4 displaying the results of extraction with KCl and Fig. 5 those of extraction with KI.

The results of the second series of experiments (B) are given in Fig. 6.

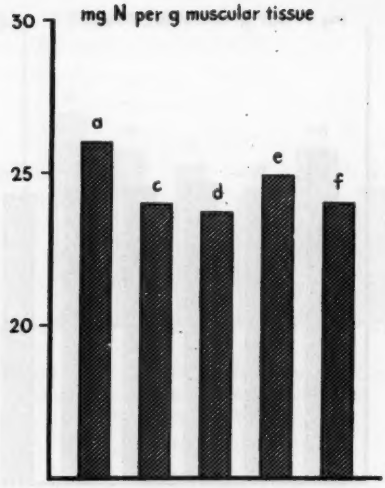

Fig. 6. Nitrogen yield using 1.1 M KI after storage of frozen sections at different temperatures.
Storage temperatures

a = − 75° C
c = + 20° C, 60 mins.
d = + 20° C, 120 mins.
e = + 8° C, 60 mins.
f = + 8° C, 120 mins.

Series A and B discloses that storage at temperatures of + 8° C and + 20° C reduces the amount of extractable nitrogen. The effect of temperature on extraction with KI solution seems to be less marked than it is on extraction with KCl solution.

Fig. 7 presents the results of the third set of experiments (C). It will be seen that muscular tissue can be stored in a deep freezer for up to 48 hours without affecting the amount of extractable nitrogen. After prolonged storage the nitrogen recovery varies inversely as the length of the storage period.

Discussion. The experiments just described demonstrate that when muscular tissue is stored at or above + 8° C the proportion of nitrogen recoverable by extraction will be reduced. Not all pre-

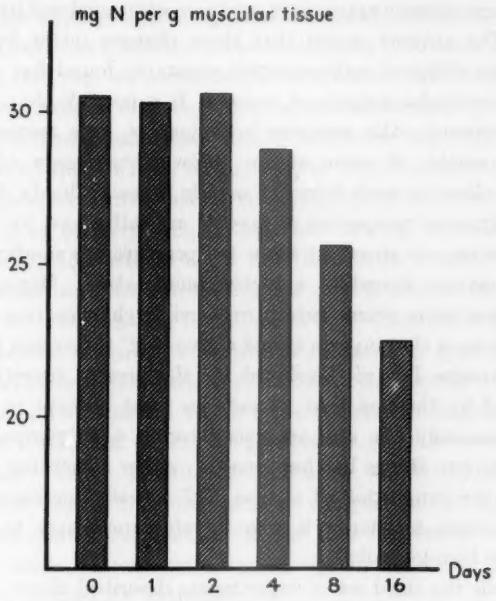

Fig. 7. Nitrogen yield using 1.1 M KI after storage of frozen sections in deep-freezer for different periods.

vious investigators have adopted preparation and extraction techniques capable of eliminating any heating of the muscle. Indeed, this could well be one reason for their lower protein recovery. Storage of tissues at moderate or high temperatures appears to cause changes that render the contractile proteins insoluble in ordinary extraction fluids. Exactly what happens is not known, but it might have something to do with denaturation or autolytic alterations. In this connection I should like to draw attention to one factor that perhaps could be responsible for the diminished recovery by extraction.

We know from LAKI & CARROLL'S (1955) findings that profound changes occur in the myosin molecule when the solution is allowed to stand at 25° to 30° C. It can be shown that storage at room temperature for 2 hours elevates the sedimentation constant of a myosin so-

lution. Longer storage apparently produces additional and irreversible changes. The authors stated that these changes might be responsible for the different sedimentation constants found for, and consequently molecular weights of, myosin. It is possible that, to quote LAKI & CARROLL, »the previous investigators were measuring the molecular weight of some stable stepwise aggregate of myosin probably isolated as such from the muscle tissue.» Clearly, therefore, the hydrodynamic properties of myosin are influenced by temperature so that myosin stored at a low temperature has smaller particle size than myosin stored at a higher temperature. Moreover it is probable that there occur certain irreversible changes, one of whose manifestations is that myosin stored above + 2° C becomes insoluble.

The technique I have developed for the present investigation is complicated by the fact that all samples must be kept in a chilled state continuously. In any analysis showing a subnormal protein yield one of two things has happened — either something had gone wrong, or the proportion of stroma had actually increased. When there was room for doubt it was therefore necessary to examine the residue histologically.

As regards the third set of experiments described above, it should be noted that the possibility of storing deep frozen muscular tissue for as long as 48 hours without manifest degradation of the recovery of extractable nitrogen solves the problem of how to store tissue when circumstances preclude immediate extraction.

5. Effect of Repeated Muscular Contractions on Protein Recovery

Introduction. As early as 1907 SAXL observed that the solubility of muscle proteins was reduced by rigor mortis or postmortal storage of the tissue. SAXL attributed this to denaturation of the myogen — which, as time would show, was an erroneous conclusion. DEUTICKE (1930) noted that less myosin could be extracted from muscles that immediately before excision had been vigorously active, for example performing repeated contractions. He also found that myosin became less soluble post mortem. These phenomena have become known as the »Deuticke effect» and have later been studied

by various authors including HENSAY (1930), MIRSKY (1936) and ERDÖS (1943). As a rule the lowered solubility of myosin is associated with the fact that an exhausted muscle sooner falls victim to rigor mortis than a rested one. Rigor mortis is characterized by disorganisation of the tissues and concomitantly adenosine triphosphate decomposes and disappears from the myofibrils. At the same rate as adenosine triphosphate disappears the solubility of myosin diminishes. Beginning at an early stage, this reduction of solubility becomes readily noticeable after 3 hours and culminates after 10—18 hours. At the same time the hardness of the muscle attains its maximum, which is attributed to the formation of an F-actomyosin gel. During the subsequent softening of the muscle it undergoes general autolysis, and meanwhile actomyosin decomposes. Apparently rigor mortis and states of contraction are in some respects analogous, as is discussed in a series of papers by DUBUISSON and his coworkers (e. g., DUBUISSON, 1950; DUBUISSON & PEZEU, 1947; JACOB, 1947 a). DUBUISSON stated that the protein composition of extracts from rested and contracted muscles differed in a number of ways. However, all these dissimilarities apparently vanish when KI or pyrophosphate is used for extraction.

The author's experiments. A rabbit was tied down to an operating table. Preoperative anaesthesia was induced with a small amount of urethane. Under local anaesthesia an incision was made through the skin over the middle of each thigh close to the sciatic nerve. The nerve was exposed and a 1 cm long section of it was removed from the left side. The nerve on the right side was merely cut off and the peripheral end stimulated again and again with 1 volt 50 c/s a. c. for about 5 minutes. As a result the calf muscles of the right leg at first contracted sharply but towards the end of the stimulation the contractions diminished until ultimately no manifest jerks could be evoked. The blood supply to both legs was never interfered with. The animal was then killed in a manner precluding further contractions (ether anesthesia). White calf muscle was excised and quickly frozen in carbon dioxide snow, sectioned with the freezing microtome and extracted in a refrigerated room for 4 hours with 10 aliquots of 1.1 M KI + 0.1 M K phosphate buffer pH 7.4.

Results. Four animals were used for the experiment. The total nitrogen and non protein nitrogen contents of the extracts were

TABLE 4. *Nitrogen yield from normal and contracted muscles of the same rabbit.*

Animal No.	Type: Normal or contracted muscle	Mg N yielded/g muscular tissue		
		Total-N	Non protein N	Protein N
1	Normal	26.7	3.2	23.5
	Contracted	27.1	3.1	24.0
2	Normal	25.9	3.4	22.5
	Contracted	25.8	3.2	22.6
3	Normal	28.7	3.3	25.4
	Contracted	27.9	3.3	24.6
4	Normal	27.9	3.0	24.9
	Contracted	28.2	3.3	24.9

determined. No differences could be observed between contracted and non-contracted muscle (cf. Table 4).

Discussion. The experiment produced no signs of the so-called Deuticke effect, the probable reason being that KI was used as solvent. Potassium iodide (cf. p. 48) depolymerizes F-actin to G-actin, and this apparently renders the contractile proteins extractable even in the absence of adenosine triphosphate.

Chapter V

ANALYSIS OF EXTRACTION TECHNIQUES

6. Effect of pH Level on Muscle Protein Extractability

Introduction. EDSALL (1930) extracted myosin at pH 8.5 to 9.0. Making systematic trials to extract myosin, GUBA & STRAUB (1943) found that pH 6.5 was optimum for high recovery. At higher pH levels, according to A. SZENT-GYÖRGYI (1944), the solubility of actin inclines while that of myosin declines. The reason for this phenomenon would seem to be that actomyosin extracted at lower pH levels contains less actin than actomyosin extracted at higher pH levels (GUBA, 1943). JACOB (1947 b) concluded from the results of his researches that 7.4—7.7 is the optimum range of pH levels and the zone of choice for extraction of the water soluble sarcoplasm proteins. Above and below pH 7.4—7.7 the total refractive increment and the total protein nitrogen both decrease. The fact that the maximal protein concentration occurs in this range is borne out by the minimal precipitation following dialysis. However, disregarding JACOB's findings, DUBUISSON (1950) and coworkers used pH levels within the range 6.5—7.0. AMBERSON *et al.* (1949) used the pH value 7.6 and HASSELBACH & SCHNEIDER (1951) pH 6.0.

The author's experiments. Frozen muscle sections were extracted in a refrigerated room for 4 hours with 10 aliquots of 1.1 M KI + + 0.1 M Britton-Robinson's universal buffer (RAUEN, 1956) the pH being varied from 3.6 to 9.7.

Results. The results of these experiments are presented in Fig. 8. The graph discloses that the protein yield was greatest in the pH range 6.5—9.0. The recovery was substantially the same anywhere within these limits.

Discussion. The pH value selected for the extractions to be performed was 7.4, the chosen value being in agreement with JACOB's findings regarding the solubility of sarcoplasm proteins. Accordingly both sarcoplasm proteins and myofibril proteins are extracted at this pH value.

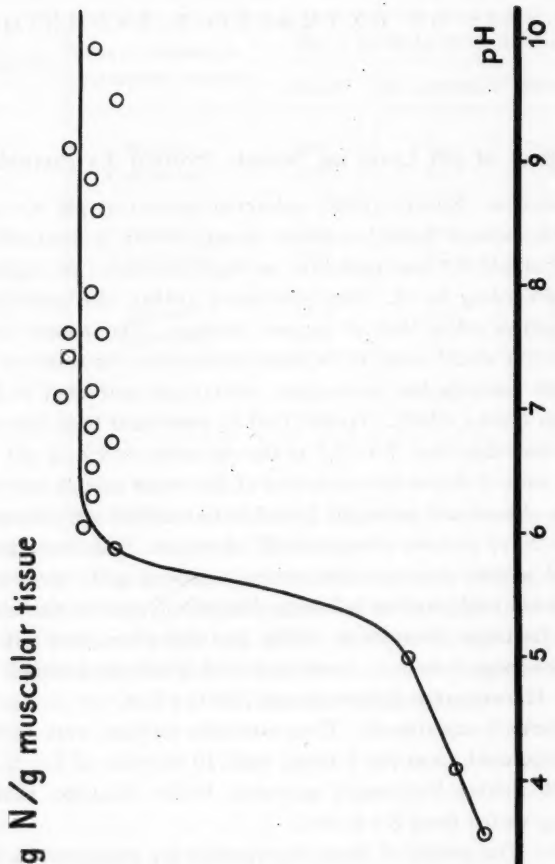

Fig. 8. Nitrogen yield using 1.1 M KI at different pH values.

Another thing disclosed by the results is that the yield is very low when extractions are carried out at pH levels on the acid side of the isoelectric point of myosin.

7. Effect of Different Salts

Introduction. While the majority of previous workers have extracted the muscular tissue in salt solutions, a few have employed organic solvents such as acetone, ether, glycerol or urea for the same purpose. Some investigators have extracted the same piece of tissue with a succession of different solutions. Extraction has in most cases been performed with potassium salts, generally the chloride (A. SZENT-GYÖRGYI, 1944) but occasionally the phosphate (AMBERSON *et al*, 1949). BATE-SMITH (1937) utilized lithium or magnesium chloride In suitable concentrations, a variety of metallic and halogen ions are capable of precipitating myosin (SZENT-GYÖRGYI, 1943). Thus, KF appears to precipitate myosin in any concentration below 0.2 M; KCl, KI and LiCl in concentrations not exceeding 0.05 M have the same result. Myosin dissolves completely in 0.2 M solutions of $MgCl_2$ or $CaCl_2$. Conversely it is completely precipitated by the same salts in concentrations not exceeding 0.1 M.

The author's experiments. A. In order to elucidate the action of various cations on the yield the following experiment was made. Muscular tissue sectioned with the freezing microtome was extracted with 1.1 M solutions of KCl, NaCl, $MgCl_2$, $CaCl_2$ and LiCl, all buffered with 0.1 M NaH_2BO_3-HCl, pH 7.4. These solutions had the same cation concentration, but the Ca and Mg salt solution had considerably higher ionic strength. Extractions were therefore also made with similarly buffered 0.367 M $MgCl_2$ and $CaCl_2$ solutions, pH 7.4, which have the same ionic strength as 1.1 M KCl.

To estimate the extractability of muscle proteins in the presence of various anions, a corresponding series was run in which frozen muscle sections were extracted for 12 hours in a refrigerated room with 1.1 M KCl + 0.1 M NaH_2BO_3-HCl buffer at pH 7.4. The yields obtained in this series were compared with the yields in similar series using KI and KBr solutions of the same molarity.

B. In order to assess the action of the iodide ion, extractions were made with different mixtures of KCl and KI, both 1.1 M + 0.1 M K phosphate buffer, pH 7.4. In this case the extraction, which took place in a refrigerated room, was discontinued after 4 hours. In this series the basis for comparison was a solution containing 100 per cent KCl and no KI. The results obtained thereby were related to the results

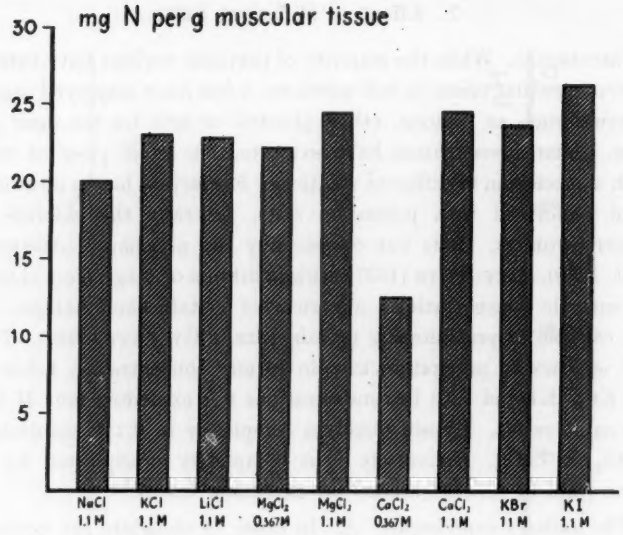

Fig. 9. Nitrogen yield using different salt solutions.

with solutions containing decreasing amounts of KCl; from 90 per cent KCl and 10 per cent KI in steps of 10 per cent to nil KCl and 100 per cent KI. Consequently the ionic strength was maintained constant.

Results. Fig. 9 presents the results of extractions with various salt solutions. (A). It will be seen that the KI solution was clearly superior to the others as an extraction agent.

The outcome of the experiments described under B appears in Fig. 10. It discloses that the yield increased with increasing KI concentrations.

Discussion. The greater extraction yields obtained with solutions of KI appear to be associated with this salt's ability to depolymerize F-actin (GUBA, 1950; SNELLMAN & GELOTTE, 1950). Since this depolymerization is irreversible, actin can be extracted in globular form. The same effect, though much less pronounced, is obtained with bromide solutions (GUBA, 1950). It should also be

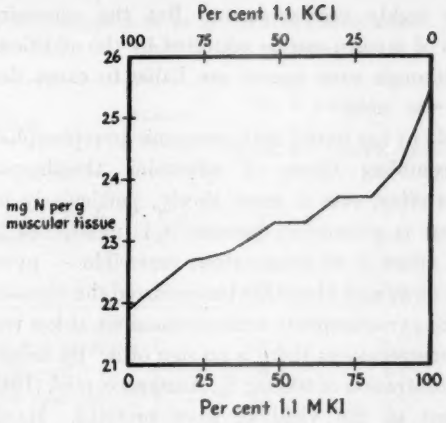

Fig. 10. Nitrogen yield using different mixtures of 1.1 M KCl and 1.1 M KI.

noted that PERRY (1953), while making extraction trials with isolated myofibrils, obtained the highest yields by the use of a solution containing KI.

Since extraction with the KI solution yielded satisfactory results it was considered unnecessary to examine the effects of still other salt solutions. Nor was it deemed justified to extract with solutions of other salts before, after or in combination with KI extraction.

8. Effect of Various Additives

Introduction. A number of investigators have attempted to increase the yield of proteins obtained from muscular tissue by adding some substance to the extraction solution. Adenosine triphosphate, the most important of these additives, is characterized by its ability to split actomyosin into its constituents actin and myosin (BANGA & A. SZENT-GYÖRGYI, 1941). When it is desired to dissolve the contractile proteins, one can accordingly reduce molecular size and viscosity by adding adenosine triphosphate to the extractant. However, owing to the pronounced adenosine triphosphatase activity of myosin, the effect of adenosine triphosphate is transient so that the extract

soon becomes highly viscous again. But the adenosine triphosphatase action of myosin can be inhibited by the addition of certain substances, although such agents are liable to cause denaturation and to reduce the yield.

STRAUB (1943 b) has found that unorganic pyrophosphates possess properties resembling those of adenosine thriphosphate. The pyrophosphate effect sets in more slowly, particularly in low concentrations, but is permanent because it is unaffected by myosin. However, the effect is to some extent reversible — pyrophosphate can be washed away and when that has occurred the viscosity increases once more. The pyrophosphate action is manifest at low temperatures only; at high temperatures there is no sign of it. By using pyrophosphate in a concentration of 400 mg %, AMBERSON *et al.* (1949) reported an improvment of the yield of fibre proteins. HASSELBACH & SCHNEIDER (1951) likewise used pyrophosphate. Organic triphosphates, calgon (ACS *et al.* 1949), guanidine and arginine (MOMMAERTS, 1945) are also able to reduce the viscosity of an actomyosin solution Urea has been proved capable of breaking up the myosin molecule into five units with approximate molecular weight 165,000 (TSAO, 1951, 1953). Furthermore, HASSELBACH & SCHNEIDER (1951) have stated that the use of urea as an additive tends to enhance the yield of actin.

Detergents have, so far as I can find, not been utilized for the purpose of increasing the yield of muscle proteins, although such substances have found a place in the extraction of nucleotides (NEURATH & BAILEY, 1953).

The author's experiments. Muscular tissue sectioned with the freezing microtome were extracted in a refrigerated room for 4 hours with 10 aliquots of 1.1 M KI + 0.1 M K phosphate buffer, pH 7.4 to which had been added in three separate series:

a. 500 mg% sodium pyrophosphate,

b. 100 mg% adenosine triphosphate, and

c. 0.1 ml 0.1 per cent Hostapal solution[1]).

The basis for comparison was a fourth series where none of these additives was employed.

[1]) Hostapal is a polyglycol ether of an alkyl phenol whose alkyl complex contains over 6 carbon atoms (supplied by Farbwerke Hoechst, Frankfort, West Germany).

TABLE 5. *Nitrogen yield using 1.1 M KI alone and in combination with certain additives.*

Solution	Animal No.	Mg N yielded/g muscular tissue		
		Total-N	Non protein N	Protein N
1.1 M KI + 0.1 M K phosphate buffer pH 7.4	1	25.9	3.1	22.8
	2	26.1	3.0	23.1
	3	25.7	3.2	22.5
	4	27.8	3.3	24.5
1.1 M KI + 0.1 M K phosphate buffer pH 7.4 + 500 mg per cent pyrophosphate	1	26.3	3.3	23.0
	2	25.6	3.1	22.5
	3	25.7	3.2	22.5
	4	27.4	3.1	24.3
1.1 M KI + 0.1 M K phosphate buffer pH 7.4 + 100 mg per cent ATP	1	26.2	3.4	22.8
	2	26.6	3.5	23.1
	3	25.9	3.4	22.5
	4	28.1	3.5	24.6
1.1 M KI + 0.1 M K phosphate buffer pH 7.4 + Hostapal	1	25.2	3.3	21.9
	2	25.5	3.1	22.4
	3	25.8	3.1	22.7
	4	26.9	3.3	23.6

Results. These experiments disclosed no differences between the yields of extractions with 1.1 M KI only and the yields of extractions with 1.1 M KI admixed with adenosine triphosphate, pyrophosphate or Hostapal solution. (Table 5).

Discussion. That the yield of extraction would be enhanced by admixture of any of the tested substances was *a priori* hardly to be expected. As mentioned previously, the use of KI extraction solution is accompanied by depolymerization of F-actin to G-actin. Accordingly the extracted actin will be in globular form. Moreover KI solvent most likely dissociates actomyosin; and, therefore, the addition of adenosine triphosphate or pyrophospate did not increase the solubility.

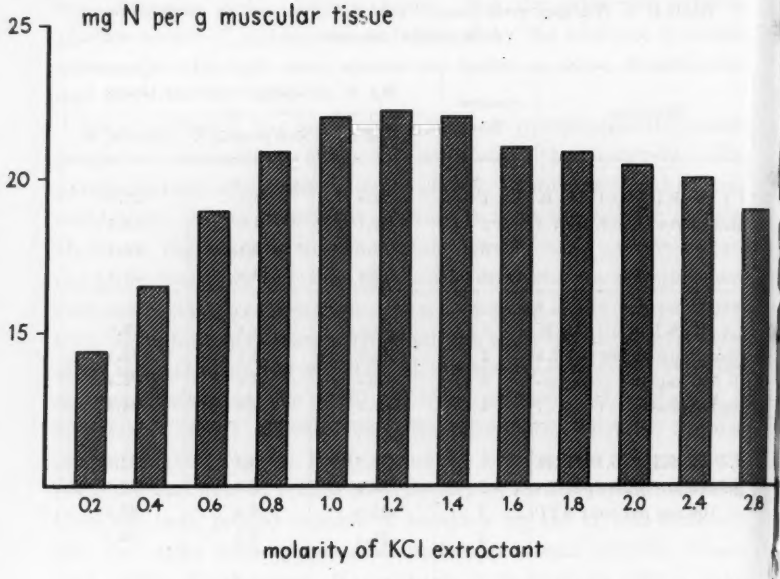

Fig. 11. Nitrogen yield using KCl solutions of different molarity.

9. Effect of Varying Ionic Strength

Introduction. It is known that myoalbumin and myogen are water soluble *in vitro* while globulin X and actomyosin dissolve at ionic strengths 0.05 and 0.3 respectively (WEBER, 1950). For the purpose of extracting contractile proteins previous investigators have generally used ionic strengths in the range μ 0.5 to 0.6 (A. SZENT-GYÖRGYI, 1944). DUBUISSON (1950) stated that maximum yield is obtained when extraction is done at ionic strengths between 0.5 and 1. It seems, however, as though DUBUISSON and his school only exceptionally extracted with solutions whose ionic strength exceeded 0.5. Moreover, in most investigations by these authors, the very first treatment applied to the product thus extracted was dialysis against a solution with ionic strength 0.15 so that some of the proteins were precipitated (JACOB, 1947 b). A dialyzed solution of this type contains little or no contractile proteins.

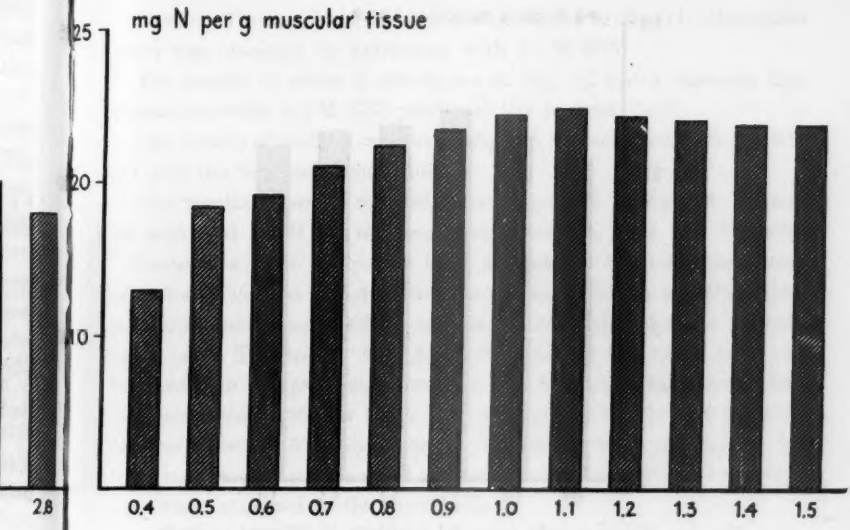

Fig. 12. Nitrogen yield using KCl solutions of different molarity.

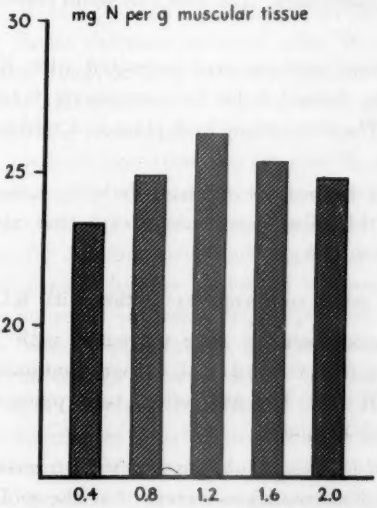

Fig. 13. Nitrogen yield using KI solutions of different molarity.

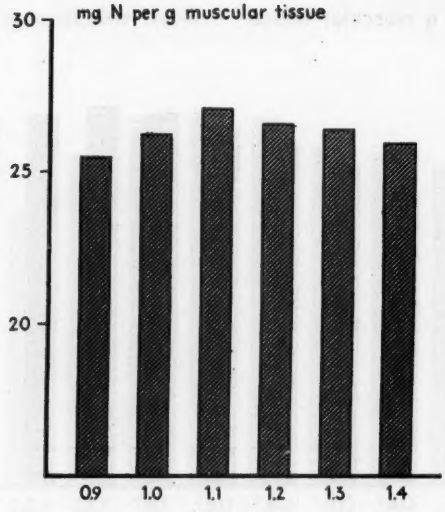

Fig. 14. Nitrogen yield using KI solutions of different molarity.

The author's experiments. The first two series concerned extraction with KCl:

a. Frozen tissue sections were extracted with KCl solutions of molarity varying from 0.2 to 2.8, containing 0.1 M K phosphate buffer pH 7.4. The extractions took place in a refrigerated room and lasted 12 hours.

b. In view of the results obtained under a, a second KCl series was run under identical conditions except that the molarity was varied from 0.4 to 1.5.

The last two series concerned extraction with KI:

c. Frozen tissue sections were extracted with KI solutions of molarity varying from 0.4 to 2.0, all of them containing 0.1 M K phosphate buffer pH 7.4. The extractions took place in a refrigerated room and lasted 12 hours.

d. In view of the results obtained under c, a second KI series was run under identical conditions except that the molarity was varied from 0.9 to 1.4.

Results. The results of series a are presented in Fig. 11. Maximum yield was obtained by extracting with 1.2 M KCl.

The results of series b are shown in Fig. 12 which discloses that extraction with 1.1 M KCl produced the highest yield.

The results of series c appear in Fig. 13, demonstrating that 1.2 M KI was the best extraction fluid.

The results of series d, lastly, are displayed in Fig. 14. It will be seen that 1.1 M KI solution gave maximum yield. (Cf. Table 6).

Discussion. The compound ionic strength of the solutions giving maximal yield was ~ 1.3. Previous instances where equally strong solutions have been used for muscle protein extraction are exceedingly rare. The reason why ionic strengths of this order have not been used in the past might well be that too much significance has been attached to the far lower ionic strength at which fibre proteins become soluble. Notably, however, the latter ionic strength is far from high enough for maximal yield of fibre proteins which seemingly are firmly attached to the myofibrils.

10. Effect of Duration of Extraction Process

Introduction. Banga & Szent-Györgyi (1941) have demonstrated that muscular tissue extracts obtained after 20 minutes are less viscous than those obtained after 24 hours. Myosin begins to dissolve within 5 minutes and initially its concentration rises. For a while the solution contains myosin alone but soon the myosin concentration begins to diminish and after 160 minutes there is no more free myosin. Concomitantly the actomyosin concentration goes up. Consequently extraction for 20 minutes yields nothing but myosin while extraction for 3 hours yields nothing but actomyosin. These phenomena are caused by the action of adenosine triphosphate combined with myosin's adenosine triphosphatase activity.

In order to ensure quantitative protein recovery, Amberson *et al.* (1949) extracted for a considerable length of time, in fact more than a week; whereas Dubuisson (1950) used fairly short periods — 30 to 90 minutes — with the same object in view. Hasselbach & Schneider's (1951) extraction periods were comparatively short, but their practice of extracting repeatedly made the total period very long so that it actually comprised 50 hours or so. Huxley

TABLE 6. *Nitrogen yield using KI solutions of different molarity.*

Molarity KI	Animal No.	Mg N yielded/g muscular tissue		
		Total-N	Non protein N	Protein N
0.9	1	25.2	3.1	22.1
	2	26.3	3.0	23.3
	3	26.0	3.3	22.7
	4	25.0	3.1	21.9
	Average	25.6	3.1	22.5
1.0	1	26.2	3.1	23.1
	2	27.0	3.2	23.8
	3	26.2	3.3	22.9
	4	25.4	3.2	22.2
	Average	26.2	3.2	23.0
1.1	1	26.9	3.4	23.5
	2	27.0	3.2	23.8
	3	28.0	3.1	24.9
	4	26.1	3.1	23.0
	Average	27.0	3.2	23.8
1.2	1	26.8	3.0	23.8
	2	27.0	3.1	23.9
	3	27.1	2.8	24.3
	4	26.1	3.2	22.9
	Average	26.8	3.0	23.7
1.3	1	26.4	3.0	23.4
	2	26.8	2.8	24.0
	3	27.3	2.9	24.4
	4	26.0	3.0	23.0
	Average	26.6	2.9	23.7
1.4	1	25.3	3.1	22.2
	2	26.9	2.9	24.0
	3	26.6	3.0	23.6
	4	24.7	3.1	21.6
	Average	25.9	3.0	22.9

TABLE 7. *Nitrogen yield using different extractants for various periods.*

Hours extracted	Mg total N obtained with			
	0.03 M phosphate	0.6 M KCl	1.1 M KCl	1.1 M KI
1	9.9	10.1	20.5	24.3
2	10.2	11.7	20.6	24.9
3	10.3	13.3	22.0	25.7
4	10.1	16.6	22.0	25.6
5	—	—	21.9	25.2
6	—	18.1	21.8	25.1
7	10.2	18.2	21.8	25.3
10	—	18.7	22.0	25.0
12	10.4	18.8	—	—
20	—	—	21.9	24.8
30	—	17.8	—	—
36	—	18.2	—	—
48	—	17.8	—	—
72	—	17.8	—	—
96	—	20.8	—	—

& HANSON (1957) extracted with glycerol for prolonged periods of the order of 2—30 weeks.

In the following experiment extraction of sarcoplasm proteins have been performed with buffer solutions of ionic strength 0.08, which corresponds to strengths of recommended solutions. (PERRY 1952).

The author's experiments. Frozen muscle sections were extracted in a refrigerated room with the four different extraction solutions specified below. Samples of the extracts were taken after different lengths of time, as described under results.

a. Extraction with 0.03 M K phosphate buffer, pH 7.4. This solution extracts sarcoplasm proteins (ionic strength 0.08).

b. Extraction with 0.6 M KCl + 0.05 M K phosphate buffer pH 7.0. This solution is typical of previous practice.

c. Extraction with 1.1 M KCl + 0.1 M K phosphate buffer, pH 7.4.

d. Extraction with 1.1 M KI + 0.1 M K phosphate buffer, pH 7.4.

It may be mentioned that whenever the extraction period exceeded 24 hours the extracts obtained were highly viscous and therefore the removal by centrifugation of residual tissue became extremely difficult.

Results. Each group comprised 3 experiments. Means of the results are given in Table 7.

As appears from the table, the sarcoplasm proteins seemed to be extracted quantitatively with 0.03 M K phosphate buffer for 2 to 3 hours. While extraction with 1.1 M KCl or KI was complete after 3 hours, the yield with 0.6 M KCl required 10 hours to reach maximum. The yield with 0.6 M KCl was less than with 1.1 M KCl whose yield in turn was less than with 1.1 M KI.

Discussion. The foregoing experiments reveal that optimally constituted solvents permit the use of short extraction periods. And short extraction periods are necessary to prevent postmortal changes in the muscle. When extraction is continued for several days or months autolysis of the tissue may set in. This might in turn release nitrogenous products from the stroma and consequently artificially augment the nitrogen yield.

11. Effect of Solvent Volume

Introduction. Needless to say the protein yield is dependent on the volume of solvent. However, the aim of most previous investigators has been to extract fibre proteins, especially myosin. This substance has usually been obtained as a precipitate by heavy dilution in order to reduce the solution's total ionic strength. Under such circumstances it is natural that A. SZENT-GYÖRGYI (1944) recommended 3 volumes of extraction solution for one volume of muscular tissue. DUBUISSON (1950) used 1.5—3 aliquots of solvent and AMBERSON *et al.* (1949) 1—2 aliquots.

The author's experiments. Two series of tissue samples were extracted for 12 hours with 2—30 volumes of extraction fluid, the solvents used being:

a. 1.1 M KCl + 0.1 M K phosphate buffer pH 7.4,

b. 1.1 M KI + 0.1 M K phosphate buffer pH 7.4.

Results. The results of these experiments are plotted in Fig. 15. It appears that the yield rises with rising solvent volume but ratios over 10: 1 have little effect upon the yield.

Discussion. The object of these experiments was to ascertain the optimum ratio of solvent volume to tissue volume, the result being

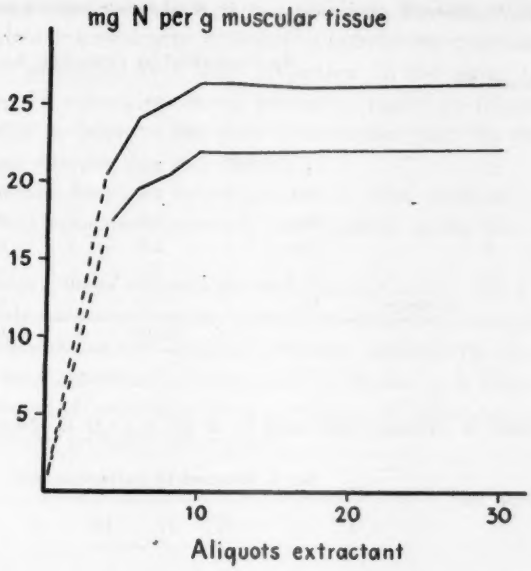

Fig. 15. Nitrogen yield using different aliquots of extractant.

that the volumes of extraction solution and muscular tissue should be in the ratio 10: 1. Considering that this ratio differs considerably from previous practice and that other significant parameters also have been far from optimal, it is understandable that serious errors have affected previous estimates of the amounts of sarcoplasm and myofibril proteins in muscular tissue.

12. Effect of Repeated Extraction on Protein Yield

Introduction. Aiming to exhaust the soluble proteins from muscular tissue, BATE-SMITH (1937) and HASSELBACH & SCHNEIDER (1951) extracted repeatedly, up to 9 times in succession. In such cases the ratio of total solvent volume to tissue volume has been of the order 30: 1 or 40: 1.

The author's experiments. Muscular tissue sections were extracted in a refrigerated room, the extractions being repeated until no more

TABLE 8. *Nitrogen yield using* 0.03 *M K phosphate buffer repeatedly.*

Animal No.	Mg N obtained by extraction No.		
	I	II	III
1	11.3	1.1	0.0
2	11.4	1.2	0.0
3	10.5	0.9	0.0
4	11.0	1.2	0.0
5	11.1	1.0	0.0
Average	11.1	1.1	0.0
		Total	12.1

TABLE 9. *Nitrogen yield using* 1.1 *M KI* + 0.1 *M K phosphate.*

Animal No.	Mg N obtained by extraction No.			
	I	II	III	IV
1	25.5	3.5	0.5	0.0
2	26.5	3.4	0.6	0.0
3	25.7	2.8	0.4	0.0
4	26.2	3.1	0.5	0.0
5	26.4	3.1	0.3	0.0
Average	26.1	3.2	0.5	0.0
			Total	29.8

nitrogenous products could be obtained. In the two series run the first two extractions lasted 3 hours and subsequent extractions 2 hours each. The extractions were consistently carried out with 10 volumes of the following solutions:

a. 0.03 M K phosphate buffer pH 7.4,
b. 1.1 M KI + 0.1 M K phosphate buffer pH 7.4.

Results. The results of series a are given in Table 8. It appears that 2 successive extractions totalling 6 hours exhausted the tissue of sarcoplasm proteins. A subsequent third extraction was tentatively prolonged for 16 additional hours, but the yield did not improve (this implies that the yield was < 0.04 mg nitrogen per g of tissue).

The results of series b are given in Table 9. To obtain a maximal protein yield it is evidently sufficient to extract the muscular tissue 3 times in succession for 8 hours altogether. In this series, too, prolongation of a subsequent fourth extraction period by 16 additional hours failed to improve the yield (this implies that the yield was < 0.04 mg nitrogen per g of tissue).

Non protein nitrogen determinations in both series of extracts showed that measurable amounts were present in the first extract only.

Discussion. These experiments demonstrated that 2 and 3 extraction periods were adequate for exhaustive extraction of respectively sarcoplasm proteins and myofibril proteins. Accordingly the corresponding total extraction periods could be limited to 6 hours and 8 hours. Repeated extractions have a washing-out effect but genuine extraction also probably takes place.

Chapter VI.

EXAMINATION OF THE PROTEIN EXTRACT

13. Protein Composition

Introduction. The various experiments detailed in Chapters IV and V were designed to provide data for optimizing the experimental conditions during extraction of muscle proteins. One parameter after another was varied and that combination selected which would produce a maximum yield of proteins. In the light of what the literature tells us about the physical and chemical properties of muscle proteins, it seems reasonable to suppose that any subnormal yield would be due to incomplete recovery of fibre proteins. It would naturally be most interesting if the protein composition of the extracts obtained in each separate experiment could be specified, because then a more penetrating analysis of the results would be possible.

Such an investigation would be most elaborate, however, partly owing to the high number of proteins existing in the muscle cell, partly because some of these proteins are not defined in the literature with sufficient accuracy for them to be readily separated and identified.

I have tried various methods for separating the proteins and for determining their concentrations in the extracts. However, paper electrophoresis was unsuitable because the paper adsorbed myosin, ultracentrifugation could not be used owing to the large number of components and salting out was ruled out due to procedural complexity. The most attractive procedure seemed to be electrophoretic separation in the Tiselius apparatus, the results of which will be described briefly in the following.

Numerous authors have made electrophoretic analyses of the protein composition of extracts containing sarcoplasm proteins and, mainly as a result of JACOB's (1947) researches, it has been possible to define fairly adequately, and to determine quantitatively, the constituents obtained (cf. p. 20).

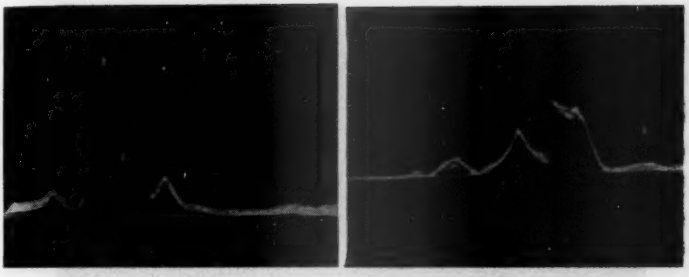

Fig. 16. Electroforetic diagram of extract obtained with 0.03 M K phosphate buffer. 180 mins. $\Theta = 45.3°$. 10 m A. The myoalbumin peak has migrated out of the view field.

I have been unable to find any papers on electrophoretic analyses of muscular tissue extracts containing the full spectrum of soluble proteins. However, extracts containing both sarcoplasm proteins and myofibril proteins have been analyzed by a series of investigators (e. g., HAAN, 1952, 1953). However, the extracts employed have been deficient in contractile proteins and therefore unrepresentative of the muscle cell's quantitative protein composition.

The author's experiments. A. Muscular tissue sections were extracted in a refrigerated room 2 times in succession for altogether 6 hours with 0.03 M K phosphate buffer pH 7.4. The resulting extracts were dialyzed against 0.04 M K phosphate buffer pH 7.4, whereupon electrophoresis was carried out.

B. Muscular tissue sections were extracted in a refrigerated room 3 times in succession for 8 hours with 1.1 M KI + 0.1 M K phosphate pH 7.4. The extracts were dialyzed against 0.15 M NaCl + 0.04 M K phosphate buffer, pH 7.4. This precipitated some of the myosin which was removed by centrifugation. The supernatant was subjected to electrophoresis.

Results. A. Electrophoresis resulted in separation of 4 different components that could be identified with JACOB's (1947 b) components, *viz.* myoalbumin and myogen (cf. Fig. 16). Neither myosin nor actomyosin was observed.

B. Electrophoresis in this case separated several components (cf. Fig. 17). In addition to those obtained in the preceding series, peaks corresponding to the myofibril proteins appeared (JACOB

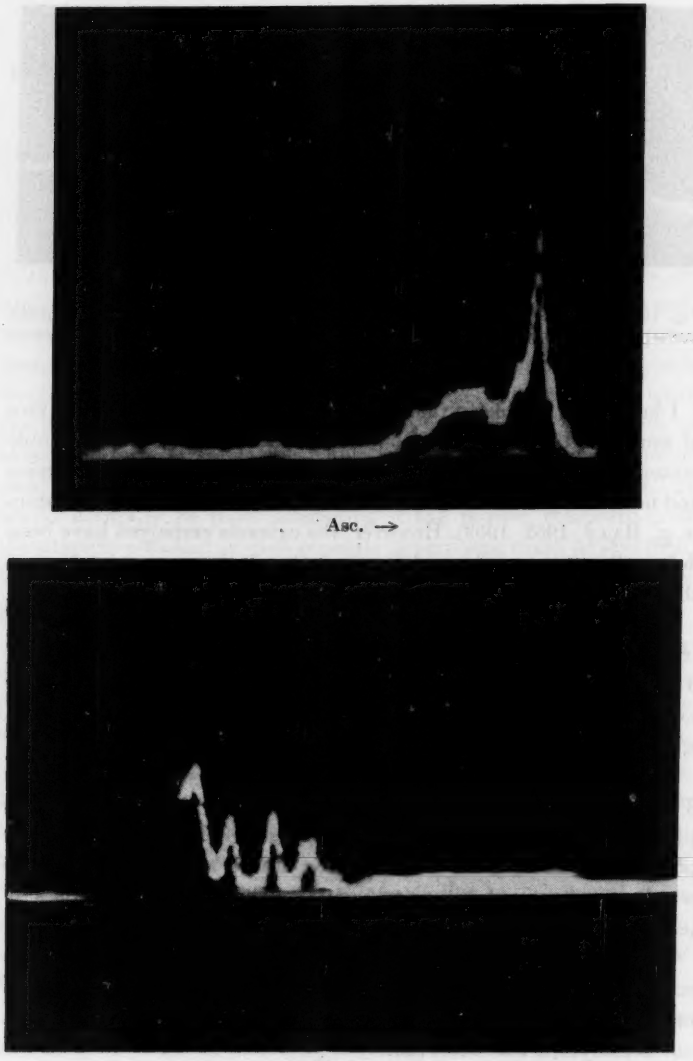

Fig. 17. Electroforetic diagram of extract obtained with 1.1 M KI + 0.1 K phosphate buffer. 280 mins. $\Theta = 38.7°$ (asc.), 45.3° (desc.) 35 m A.

1947 b). The electrophoretic diagrams showed in this case convections.

Discussion. The results obtained are compatible with the corresponding findings of previous investigators. Myosin and actomyosin are too insoluble at ionic strength 0.08 for them to appear in electrophoretic diagrams.

14. Viscosimetric Analysis

Introduction. Prolonged extraction of muscular tissue yields no myosin but only actomyosin (A. SZENT-GYÖRGYI, 1944), as was explained in section 10. Adenosine triphosphate is capable of breaking up actomyosin into actin and myosin (BANGA & SZENT-GYÖRGYI, 1941). One consequence of this is reduced viscosity.

The author's experiments. Muscular tissue sections were extracted in a refrigerated room for 3 hours with 0.03 M K phosphate buffer pH 7.4. The viscosity of the extracts was determined before and immediately after addition of 0.1 per cent adenosine triphosphate.

Results. The extracts showed the same viscosity before and after addition of adenosine triphoshate.

Discussion. This experiment suggests that extracts intended to contain sarcoplasm proteins alone were free from actomyosin.

15. Microscopic Examination

Introduction. Researches by several cytologists — e. g. SCHNEIDER *et al.* (1948) and HOGEBOOM *et al.* (1948) — have disclosed that such cell structures as mitochondria, nuclei, granulae and microsomes are capable of remaining freely suspended in a solution but are caused to sediment by centrifugation. After careful examination of various fractions of these particles, PERRY & HORNE (1952) concluded that, in rabbit muscles, nuclei and granulae occupy only a minor portion of the total cell volume.

The author's experiments. Muscular tissue sections were extracted for 3 hours in a refrigerated room with 0.03 M K phosphate buffer in one series and with 1.1 M KI + 0.1 M K phosphate buffer in another series, both solutions at pH 7.4. The extracts were then

centrifuged at 1,400 g for 20 minutes before being examined under the phase contrast microscope.

Results. Systematic examination of several extracts disclosed the presence of solitary, extremely thin, round or rectangular fragments, their number not exceeding one per field of the microscope. 0.2 ml samples from numerous such extracts contained 3 or 4 of these fragments.

Discussion. The microscopic examination furnished evidence indicating that the extracts contained so very few suspended cell fragments that they scarcely could artificially increase the amount of extractable nitrogenous substances.

16. Examination of Centrifuged Extracts

Introduction. Freely suspended granulae, mitochondria, etc. can, as mentioned in the preceding section, be made to sediment through high-speed centrifugation. PERRY & HORNE (1952) stated that the granular fraction from muscular tissue can be separated into portions containing heavy and light granulae. The heavier particles are thrown down after 10 minutes at 1,000 g whilst the lighter only sediment after 60 minutes at 19,000 g.

The author's experiments. Tissue sections were extracted in the usual manner with 0.03 M K phosphate buffer, pH 7.4. Extracts were first centrifuged for 20 minutes at 1,400 g. Samples were then taken for determination of nitrogen content. The same extracts were now centrifuged once more for 20 minutes at 25,000 g, whereupon fresh nitrogen determinations were made.

The extracts centrifuged at high speed were compared under the phase contrast microscope with those centrifuged at 1,400 g.

Results. This experiment disclosed no differences in the nitrogen content of the two extracts centrifuged at different speeds nor in their appearance under the phase contrast microscope.

Discussion. The investigation makes it clear that the extracts did not contain either microscopically visible or submicroscopical freely suspended particles in sufficient numbers to influence the nitrogen content of the extracts.

Chapter VII

EXAMINATION OF THE MUSCULAR TISSUE RESIDUE AFTER EXTRACTION

In the investigations described in this chapter the muscles were excised in the usual manner, sectioned with the freezing microtome and extracted 3 times in succession for 8 hours all in a refrigerated room with 10 aliquots of 1.1 M KI + 0.1 M K phosphate buffer pH 7.4. After the extracts had been centrifuged and the supernatant decanted, the tissue residue was examined. Apart from nitrogen determinations in the muscle residue to make sure that the nitrogen quantity left was in conformity with the nitrogen quantity originally present in the muscular tissue minus the extracted — the following histological and chemical examinations have been performed.

17. Histological Examination of the Muscle Residue

Introduction. WEBER & MEYER (1933) found that the tissue residue remaining after these authors' extraction experiments exhibited histologically manifest muscle cell remnants, among other things in the shape of unobliterated striation. It follows that their extraction cannot have been exhaustive.

HASSELBACH & SCHNEIDER (1951) stated that electron microscopical examination of the muscular residue disclosed only connective tissue structures. But this is not conclusive, for electron microscopy is not an ideal method of ascertaining the presence or absence of cell remnants. The amount of matter that can be inspected at one time is so small that very large series would be required to establish the absence of muscular tissue.

The author's experiments. The tissue was dialyzed against an adequate volume of distilled water so that the extraction solution would be substantially removed. The residue was then fixed in 10 per cent formalin, embedded in paraffin and cut into sections

5—10 μ thick. Some unstained sections were examined with a polarization microscope. Two further series were examined in an ordinary microscope, one being stained by Delafield's method, the other stained with iron-alum-haematoxylin.

Results. Examination of the residue in no case disclosed the presence of muscular tissue. Striation or double refraction could not be observed anywhere.

Discussion. These experiments strongly suggest that the tissue residue was wholly free from extractable muscular tissue.

18. Re-extraction of Tropomosin from the Muscle Residue

Introduction. Previous investigators have not, so far as I can find, re-extracted the muscle residue, if exception be made for BATE-SMITH (1937) who extracted with 0.01 N HCl and HASSELBACH & SCHNEIDER (1951) who used urea. Their re-extraction resulted in a nitrogen yield that was taken into account in their final figures for the protein yield.

The author's experiments. Initially 15 g of muscle were sectioned and extracted. Then tropomyosin was extracted by BAILEY's (1948) method. The tissue was mixed with 15 ml of redistilled water and allowed to stand at room temperature for 30 minutes. The juice was then squeezed out and 15 ml of 97 per cent ethanol added to the residue. The liquor was again expressed and the muscle residue treated with a 1:1 mixture of ethanol-water. Washing was continued through two changes of ethanol and two of ether. The fibres were now dried at room temperature and, while still damp with ether, immersed in 100 ml of M KCl. The pH was adjusted to 7 with N NaOH. After standing for 12 hours at 18° C, the supernatant was filtered off.

The protein content of the KCl extract was evaluated by means of nitrogen determinations.

Results. Addition of trichloroacetic acid to the extract produced no precipitate. Kjeldahl analysis of 5 ml of the extract disclosed no nitrogen. This implies that the amount of tropomyosin extracted cannot have exceeded 0.06 mg per g of muscular tissue.

19. Re-extraction of Actin from the Muscle Residue

The author's experiments. For this investigation 15 g of tissue were sectioned with the freezing microtome and extracted. Actin was extracted from the muscle residue according to STRAUB (A. SZENT-GYÖRGYI, 1951). The muscle residue was suspended in a 75 ml of a 0.4 per cent solution of $NaHCO_3$ and kept at a constant temperature of 22° C for 30 minutes under constant stirring. The supernatant was filtered off and the residue suspended in 15 ml of cold 0.01 M $NaHCO_3$ + 0.01 M Na_2CO_3 solution. The temperature was maintained at + 8° C and the suspension stirred thoroughly. After 10 minutes the suspension was diluted with 150 ml of glass-distilled water at 22° C. The fluid was then removed by centrifugation and 15 ml of acetone added to the residue. After stirring for 10 minutes the acetone was pressed out through a cheesecloth. To this residue 15 ml of acetone was again added, and after stirring for 10 minutes the fluid was expressed. The solid matter thus obtained was dried at room temperature for 10 hours.

The acetone dried muscle residue was extracted with 8 ml of CO_2-free, glass-distilled water for 20 minutes at room temperature. Lastly, after centrifugation, the protein content of this extract was evaluated by means of nitrogen determination.

Results. Addition of trichloroacetic acid yielded no precipitate. The presence of nitrogen could not be detected by Kjeldahl analysis of 2 ml of the extract. This implies that, after application of the present author's method, the amount of actin extracted by STRAUB's technique did not exceed 0.012 mg per g of muscular tissue.

Discussion. Since, as stated above, the muscle residue contained no measurable amounts of either tropomyosin or actin, it must be taken as proved that the prior KI treatment had exhaustively extracted these substances. The fact that measurable amounts of these — the least soluble muscle proteins — could not be extracted from the muscle residue warrants the conclusion that all other more soluble proteins were exhaustively extracted.

Chapter VIII

OPTIMAL CONDITIONS FOR QUANTITATIVE PROTEIN EXTRACTION FROM SKELETAL MUSCLE

The optimal conditions for protein extraction from skeletal muscle, as disclosed by the experiments described in Chapters III to VII, may be summarized as follows.

The animal whose muscles are to be analyzed is killed without anaesthesia by a blow against the skull. Exsanguination is effected by holding the animal head downwards while the large cervical vessels are cut and the blood is running off. Immediately after exsanguination the muscles are excised and rid of fasciae, fatty tissue, nerves and vessels. Dry substance and nitrogen determinations are carried out on a representative muscle sample.

The muscular tissue is then rapidly chilled in carbon dioxide snow; and, provided the temperature is not allowed to exceed $-20°$ C, the tissues can be stored for up to 48 hours with no effects on the extractability of the muscle proteins.

The frozen tissue block is then sectioned across the fibres with the freezing microtome into slices 20 μ thick. The sections are put one by one into a vessel containing carbon dioxide snow. They are transferred from this vessel, care being taken to remove any adhering carbon dioxide snow, to Pyrex glass extraction tubes having precisely fitting stoppers. The sections are quickly weighed in these tubes, the nominal quantity per tube being 1 g. Two portions are weighed for each tissue sample. The weighed tubes are immediately placed in carbon dioxide snow in order to preclude any thawing before the extraction can begin. Extraction takes place at $+2°$ C, the samples being agitated slowly all the time.

One of the duplicate samples is extracted with 10 aliquots of 0.03 M K phosphate buffer pH 7.4 for 3 hours and then centrifuged for 20 minutes at 1,400 g. The supernatant is saved and the muscle residue re-extracted with the same solvent under the same conditions

as before. After renewed centrifugation the sarcoplasm proteins will be exhaustively removed from the muscle residue. The total nitrogen content of the extracts is determined. Then trichloroacetic acid is added to an extract sample to precipitate the proteins whereupon an assay of the non protein nitrogen content is made. From the figures obtained the amount of sarcoplasm proteins can readily be calculated.

The tube with the second of the duplicate muscular tissue samples is extracted with 10 aliquots of 1.1 M KI + 0.1 M K phosphate buffer, pH 7.4 for 3 hours and centrifuged exactly as above. The extract is saved and the muscle residue extracted with the same solution for another 3 hours. After centrifugation the extract is saved and the residue extracted a third time with the same KI solution for 2 more hours. Both sarcoplasm and myofibril proteins will now be exhaustively extracted from the muscular tissue. The total nitrogen content and, after protein precipitation with trichloroacetic acid, the nonprotein nitrogen content of the extracts are now determined, whereupon the protein nitrogen content can be deduced. After calculation of the quantity of dissolved protein nitrogen and subtraction of the figure obtained for sarcoplasm proteins, the result obtained will denote the amount of myofibril proteins.

The nitrogen content of fresh muscle, the quantities of sarcoplasm and myofibril proteins and the amount of non protein nitrogen are now known. Therefore the amount of undissolved nitrogen can be deduced and used as a basis for calculation of the amount of stroma proteins.

The block diagram in Fig. 18 illustrates the main features of the procedure.

In order to illustrate the accuracy of the method, the numerical results will now be given of an analysis. The standard deviations were obtained by statistical treatment of the results of 16 analyses of identical samples of a selected portion of a rabbit's thigh muscles having no fasciae.

	Per cent
Water	76.15
Dry Substance	23.85

	Mg N per g fresh tissue
Total Nitrogen	32.7 ± 0.14
Nitrogen extracted with 1.1 M KI + 0.1 M K phosphate buffer, pH 7.4	30.9 ± 0.20
Nitrogen extracted with 0.03 M K phosphate buffer, pH 7.4	13.8 ± 0.07
Non protein nitrogen	3.4 ± 0.05

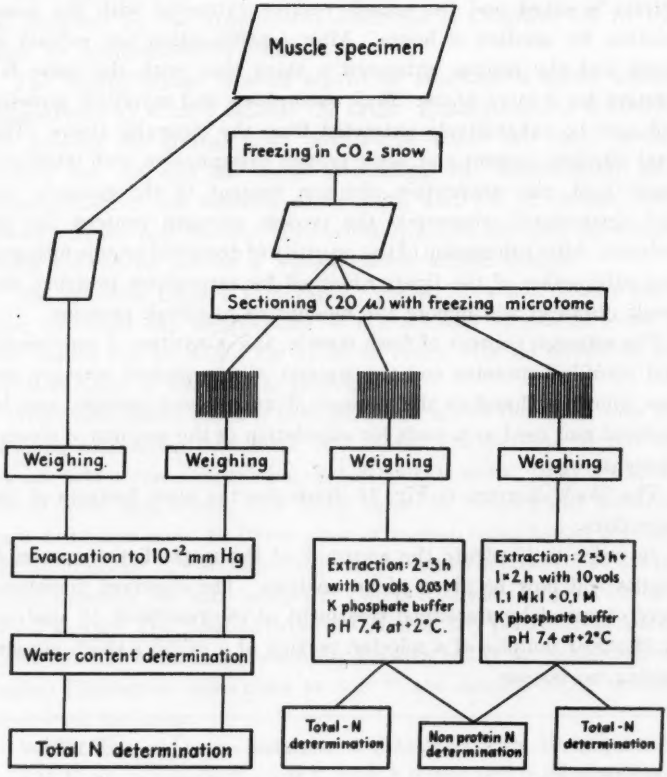

Fig. 18. Block diagram showing main features of the author's technique for analysis of muscle tissue.

It will be realized that in this analysis 94.6 ± 0.61 per cent of the nitrogen content of the tissue was extracted. From the figures obtained the several nitrogen fractions can be calculated.

	Mg N per g fresh tissue	Per cent of total N
Myofibril proteins	17.1 ± 0.21	52.3 ± 0.64
Sarcoplasm proteins	10.4 ± 0.08	31.8 ± 0.24
Non protein nitrogen.....	3.4 ± 0.05	10.4 ± 0.15
Stroma protein	1.8 ± 0.24	5.5 ± 0.73

Alternatively the amounts of the protein fractions can be expressed in grammes per 100 grammes of fresh muscular tissue. This leads to the following.

	G proteins per 100 g fresh tissue	Per cent of total protein
Myofibril proteins.....	11.03 ± 0.135	58.4 ± 0.71
Sarcoplasm proteins	6.71 ± 0.052	35.5 ± 0.28
Stroma proteins	1.16 ± 0.155	6.2 ± 0.82

Thus, in this case, about 60 per cent of the muscle proteins would seem to consist of myofibril (contractile) proteins and about 35 per cent of sarcoplasm proteins. The insoluble nitrogen fraction has here been characterized as stroma proteins and must be considered equivalent to the nitrogen content of cell walls, endomysium, perimysium, vessels and nerves. This fraction comprises between 5 and 6 per cent of the total nitrogen.

For the sake of completeness the main components of and their percentages by weight in muscular tissue are given in Table 8.

Discussion. Application of the method described in this chapter permits calculation of the sarcoplasm protein content and the myofibril protein content of muscular tissue. Formerly, as discussed in Chapter II, it has not been feasible to calculate the figures in question with resonable accuracy. The reason for the great variability of

TABLE 8. *Main components of and their percentage by weight in muscular tissue*

	Per cent
Myofibril proteins	11.03
Sarcoplasm proteins	6.71
Stroma proteins	1.16
Glucogen (DUBUISSON, 1954)	1.00
Fats (INGELMARK & HELANDER, 1951)	1.00
Salts, carnosine, etc. (DUBUISSON, 1954)	1.50
Water	76.15
Unidentifiable	1.45
Total	100.00

previous investigators' results would seem to be that they have failed to appreciate the necessity of maintaining constant experimental conditions throughout a series of analyses. This has also made it impossible to compare normal and abnormal muscular tissues.

An adequate answer to the question whether the specified sarcoplasm protein and myofibril protein quantities are proportional to the true volumes of sarcoplasm and myofibrils must await the evolution of an acceptable procedure for determining the intravital water content of these structures. Considerable differences would probably be found if contracted and relaxed muscles could be compared with respect to the intravital water content of sarcoplasm and myofibrils.

PART TWO

PROTEIN COMPOSITION OF NORMAL AND ATROPHIC MUSCLES

Chapter IX

MATERIALS AND GENERAL METHODS

Muscle specimens for the protein analyses recounted in Chapter X were obtained partly from rabbits, and partly from cattle. The pieces of cattle muscle were dissected out less than 10 minutes after the animal had been killed. In principle the exsanguination was accomplished by the same technique as for rabbits. With respect to muscle from hearts and calf foetuses it must be mentioned, however, that judging by macroscopical features the exsanguination seemed less complete than in muscle specimens of other types.

Specimens for the experiments discussed in Chapter XI consisted of white calf muscle (gastrocnemius + soleus) from rabbits similar to those described in Chapter III (p. 25). Anatomical conditions make the preparation of specimens of calf muscle more difficult than of thigh muscle from the same species, particularly with respect to the removal of fasciae.

Experiments comparing normal and atrophic muscle were done with rabbits having artificially induced atrophy. Unilateral interventions were made on the right and left sides alternately, and the results were always based on comparisons between the atrophic muscle and the contralateral normal muscle of the same animal.

Details of the surgical techniques are given in conjunction with the associated experiment. All interventions were made under anaesthesia. Urethane alone was used for application of plaster casts, urethane supplemented by ether on open mask for tenotomy and neurotomy. Neither local infections nor haemorrhages were observed during the operations or at post mortem examinations.

In each series the period of observation was 20 days. This period was chosen because, as appears from previous literature (Lipschütz & Audova, 1921; Fischer & Ramsey, 1946 a), it is long enough

for the resulting reduction of the wet weight of the muscular tissue to amount to some 25—35 cent. An atrophy of this order must be considered adequate for the subsequent analyses.

Other information on general methods is given in Chapter III. The amounts of water, total nitrogen, protein fractions and non protein nitrogen were determined in the manner described in Chapter VIII.

Chapter X

PROTEIN CONTENT OF NORMAL STRIATED MUSCLE OF VARIOUS TYPES

Introduction. Comparatively few papers deal with the variations of the protein fractions in muscles of different kinds. The proteins have in most cases been extracted at ionic strengths below 1.3, implying that the protein yields cannot have been exhaustive. Separation of the protein components has usually been accomplished by electrophoresis. (JACOB 1947 b; HAAN, 1953). A few papers deal with the concentration of single proteins in various types of muscle.

The author's experiments. Skeletal and heart muscle specimens from different animals were analyzed in accordance with the method described in Chapter VIII. The method was thus applied to tissues other than that for which it was originally developed, the object merely being to provide some information as to its general applicability.

Results. The results obtained will be found in Table 10. It reveals that different types of muscle have very dissimilar protein compositions. The number of experiments was far too small to warrant the drawing of statistically justifiable conclusions.

Discussion. From the concordant findings of numerous investigators it is well known that work and disuse both cause great changes in the locomotive apparatus. The changes in question have in most cases been interpreted as the manifest signs of a process of adaptation. The tissues adapt themselves to the degree and type of activity they are called upon to perform. This adaptation affects not only the composition of the major tissue elements but also some of the intracellular structures.

The fact that the protein composition varies from one type of muscle to another is, as I see it, the result of adaptation of each type of muscle to its particular function. Muscles on which rapid

and powerful activity makes great demands should appropriately have a relatively high content of contractile proteins. If the muscular tissue is called upon to do only little or moderate work, the need for contractile proteins should be comparatively small and, consequently, the sarcoplasm proteins would comprise a relatively larger share.

TABLE 10. *Nitrogen yield from striated muscular tissue from different regions using the author's technique*

TABLE 10. *Nitrogen yield from striated muscular tissue from different regions using the author's technique*

Animal	Muscle type	Age or weight	Mg Myofibril-protein N/g muscular tissue	Mg sarco-plasmprotein N/g muscular tissue	Mg non protein N/g muscular tissue	Mg stroma N/g muscular tissue	Water per cent
Rabbit (mean of 51 analyses)	Gastrocnemius + soleus	1—2 kg.	18.5 ± 0.25	7.9 ± 0.16	3.3 ± 0.02	2.7 ± 0.44	76.3 ± 0.14
Calf foetus	Gastrocnemius + soleus	(7 months)	9.3	7.1	1.8	9.2	79.1
Calf foetus	Gastrocnemius + soleus	(7 months)	8.2	7.8	2.0	10.0	78.7
Cattle	Gastrocnemius + soleus	5 days	14.0	6.8	2.5	5.4	77.8
Cattle	Gastrocnemius + soleus	7 days	15.5	7.2	2.8	4.5	76.4
Cattle	Gastrocnemius + soleus	6 weeks	11.6	12.5	3.0	4.0	77.2
Cattle	Gastrocnemius + soleus	6 weeks	16.0	9.0	3.3	3.2	76.1
Cattle	Gastrocnemius + soleus	2 months	13.0	7.8	2.8	5.1	77.8
Cattle	Gastrocnemius + soleus	2 months	11.9	9.7	3.0	4.3	76.4
Cattle	Gastrocnemius + soleus	1.5 years	18.2	7.9	3.4	3.4	76.2
Cattle	Gastrocnemius + soleus	11 years	16.2	9.3	3.4	3.9	75.3
Cattle	Gastrocnemius + soleus	14 years	20.2	8.2	3.2	3.0	75.7
Cattle	Tongue	7 days	11.5	6.8	2.0	12.1	75.1
Cattle	Heart auricle	5 days	7.8	6.8	1.0	13.7	75.9
Cattle	Heart auricle	7 days	9.5	4.6	1.1	14.9	77.2
Cattle	Heart auricle	2 months	8.9	5.5	1.3	15.8	76.3
Cattle	Heart ventricle	5 days	11.4	6.8	2.4	10.2	77.5
Cattle	Heart ventricle	7 days	12.4	6.1	1.9	9.8	77.1
Cattle	Heart ventricle	2 months	12.1	6.6	1.9	10.5	76.0

Chapter XI

STUDIES ON ATROPHIC MUSCLES

Introduction. A. Weight loss. All kinds of atrophy are associated with weight loss. The weight loss is most pronounced after neurotomy, slightly less marked after tenotomy and least after disuse (cf. LANGLEY & NATO, 1915; LIPSCHÜTZ & AUDOVA, 1921; CHEN *et al.*, 1924; LIPPMAN & SELIG, 1928; CHOR *et al.* 1937; FISCHER & RAMSEY, 1946 a).

B. Total nitrogen content and protein composition. AVELLONE & MACCO (1925) reported a reduction of the total nitrogen content following denervation. CAHN (1927) found that denervated muscles showed lower contents of both total nitrogen and extractable nitrogen. HINES & KNOWLTON (1933) and CHOR *et al.* (1937) were unable to verify this finding.

Previous investigations of the protein composition of atrophic muscles have been devoted partly to collagen, partly to soluble proteins.

FISCHER & RAMSEY (1946 a) demonstrated that the relative but not the absolute collagen content varied inversely as the weight loss.

The myogen fraction appears to diminish following denervation atrophy (HAAN, 1953; FISCHER, 1955). If aldolase activity is accepted as a measure of the myogen content, the latter decreases considerably (FISCHER *et al.*, 1949).

According to a consensus of opinion the myoalbumin content rises in conjunction with all kinds of atrophy (CREPAX, 1949; FISCHER *et al.*, 1949; HAAN, 1953).

Investigations into contractile protein variations demonstrate that different types of atrophy are accompanied by reduction of the myosin and actin fractions (HINES & KNOWLTON, 1933; WESTENBRINK & KRABBE, 1936; FISCHER & RAMSEY, 1946 a; HAAN, 1953). In this connection it may be mentioned that certain observations suggest that denervation atrophy gives rise to qualitative changes in the myosin molecule (DREYFYS *et al.*, 1953).

FISCHER & RAMSEY (1946 b) found that daily electrical stimulation of denervated muscles was capable of retarding the weight loss and deterioration of muscle proteins.

C. Non protein nitrogen content. GRUND (1913) stated that the non protein nitrogen content of the muscles in an amputation stump decreased. FISCHER & RAMSEY (1946 a) found a small increase of non protein nitrogen in disuse atrophy and a small decrease in atrophy induced by denervation or tenotomy.

D. Water content. The water content of atrophic muscular tissue is dealt with in a small number of publications. AUDOVA (1923) found no significant differences between normal and tenotomized or neurotomized animals, while GRUND (1912, 1913) reported a small elevation in disuse atrophy and no change in denervated muscles. FISCHER & RAMSEY (1946 a) obtained a small diminution of the water content of muscles following experimental disuse atrophy whilst an insignificant increment was noted in neurotomized and tenotomized muscles. CHOR *et al.* (1937) found no difference between the water contents of normal and neurotomized muscles.

The author's experiments. Atrophy was induced in rabbits by neurotomy, tenotomy and disuse due to immobilization in plaster casts.

A. Neurotomy was performed as follows. Via a longitudinal slit through the skin on the dorsal surface of the thigh and an incision made in the fascia the muscles were reached and moved sideways to expose the sciatic nerve. A nerve section 1.0 to 1.5 cm long about half way up the thigh was removed, whereupon the muscles were replaced and the skin sutured.

The animals were killed after 20 days and in every case it was ascertained that no connections existed between the peripheral and central nerve ends. Altogether 17 animals were neurotomized.

B. Tenotomy was performed as follows. A longitudinal incision was made along the Achilles tendon and the latter was dissected free. A 1 to 1.5 cm long section of the tendon immediately above the calcaneus was removed. The skin was sutured.

The animals were killed after 20 days and in every case it was ascertained that no connection existed between the distal end and the insertion of the tendon. Altogether 17 animals were tenotomized.

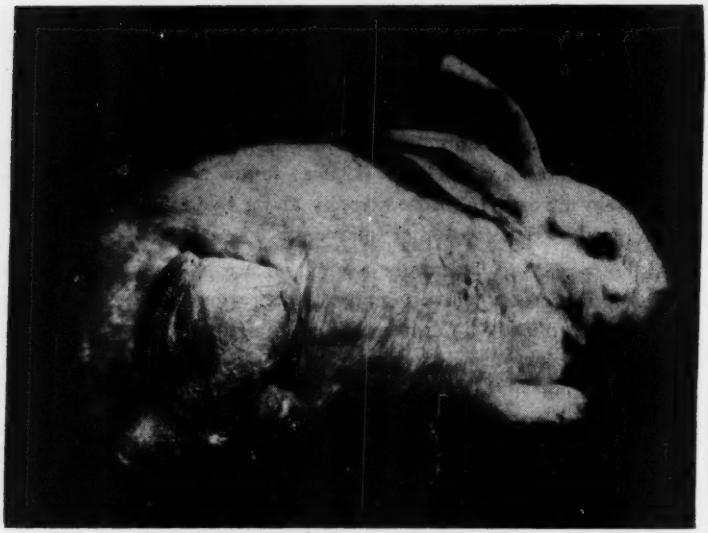

Fig. 19. Rabbit immobilized in plaster cast in the normal crouching position.

C. Disuse atrophy was induced by immobilizing with a plaster cast one of the animal's hind legs in the normal crouching position (cf. Fig. 19). Although the gastrocnemius and soleus muscles were not completely immobilized by the plaster cast, their mobility was restricted.

The animals were killed after 20 days and in every case it was ascertained that the plaster cast was correctly positioned and that the skin beneath it was uninfected. Altogether 17 animals were subjected to plaster cast immobilization.

Results. The following determinations were made on each of the 102 available muscles: degree of atrophy, dry substance content, total nitrogen content, protein composition specifying the separate myofibril, sarcoplasm and stroma protein fractions, and non protein nitrogen content.

The results appear in Tables 11 to 13. In each analysis (i. e. one animal) the atrophic calf muscles (gastrocnemius + soleus) were compared with the contralateral normal calf muscles. The total

number of normal calf muscles was 51, but separate figures are given for each of the 3 groups of 17 animals because biological variations might have introduced sources of error if all the normal muscles had been combined into a single group.

The ratio of the weights of the atrophic and the contralateral muscles was 66.9 ± 1.73 per cent after neurotomy, 67.5 ± 1.94 per cent after tenotomy and 79.0 ± 3.38 per cent after disuse.

TABLE 11. *Nitrogen yield from normal and denervated muscles of the same rabbit.*

	Normal muscle	Denervated muscle	Deviations denervated in per cent of normal
Total N mg/g muscle tissue	32.3 ± 0.42	26.0 ± 0.55	80 ± 1.6
Myofibril-N mg/g muscle tissue	18.3 ± 0.41	13.1 ± 0.43	71 ± 2.0
Sarcoplasm-N mg/g muscle tissue	8.3 ± 0.18	6.3 ± 0.22	78 ± 2.4
Non protein N mg/g muscle tissue	3.2 ± 0.04	2.8 ± 0.06	86 ± 2.0
Stroma-N mg/g muscle tissue ...	2.8 ± 0.09	4.0 ± 0.12	149 ± 4.6
Dry weight per cent	24.0 ± 0.19	23.2 ± 0.25	

TABLE 12. *Nitrogen yield from normal and tenotomized muscles of the same rabbit.*

	Normal muscle	Tenotomized muscle	Deviations tenotomized in per cent of normal
Total N mg/g muscle tissue	32.0 ± 0.32	26.4 ± 0.47	83 ± 1.5
Myofibril-N mg/g muscle tissue	18.7 ± 0.57	14.0 ± 0.65	75 ± 3.3
Sarcoplasm-N mg/g muscle tissue	7.7 ± 0.41	6.8 ± 0.31	92 ± 4.9
Non protein N mg/g muscle tissue	3.2 ± 0.04	2.8 ± 0.05	89 ± 1.9
Stroma-N mg/g muscle tissue ...	2.6 ± 0.08	3.1 ± 0.09	120 ± 5.8
Dry weight per cent	23.7 ± 0.32	23.0 ± 0.36	

TABLE 13. *Nitrogen yield from normal and immobilized muscles of the same rabbit.*

	Normal muscle	Immobilized muscle	Deviations immobilized in per cent of normal
Total N mg/g muscle tissue	32.4 ± 0.35	30.4 ± 0.55	94 ± 1.3
Myofibril-N mg/g muscle tissue	18.7 ± 0.32	14.9 ± 0.52	79 ± 2.7
Sarcoplasm-N mg/g muscle tissue	7.9 ± 0.25	8.9 ± 0.28	115 ± 2.9
Non protein N mg/g muscle tissue	3.3 ± 0.02	3.6 ± 0.04	110 ± 1.2
Stroma-N mg/g muscle tissue ...	2.7 ± 0.06	3.3 ± 0.10	122 ± 4.1
Dry weight per cent	23.7 ± 0.19	24.6 ± 0.20	

Considering now the contributions in per cent of the several nitrogen components to the total nitrogen content of each muscle, the following figures are obtained.

TABLE 14. *Nitrogen components in per cent of total nitrogen content, denervated muscles.*

	Normal muscle	Denervated muscle
Myofibril-N %	56.4 ± 0.73	49.8 ± 0.78
Sarcoplasm-N %	25.4 ± 0.55	24.3 ± 0.57
Stroma-N %	8.5 ± 0.31	15.6 ± 0.46
Non protein N %	9.9 ± 0.20	10.6 ± 0.26

TABLE 15. *Nitrogen components in per cent of total nitrogen content, tenotomized muscles.*

	Normal muscle	Tenotomized muscle
Myofibril-N %	58.5 ± 1.55	52.7 ± 1.81
Sarcoplasm-N %	24.3 ± 1.40	25.9 ± 1.37
Stroma-N %	8.0 ± 0.25	11.6 ± 0.45
Non protein N %	10.0 ± 0.14	10.6 ± 0.32

TABLE 16. *Nitrogen components in per cent of total nitrogen content, immobilized muscles.*

	Normal muscle	Immobilized muscle
Myofibril -N %	57.1 ± 0.76	47.8 ± 1.17
Sarcoplasm-N %	24.3 ± 0.74	29.2 ± 0.80
Stroma-N %	8.4 ± 0.19	10.9 ± 0.47
Non protein N %	10.0 ± 0.10	11.7 ± 0.22

The figures given in the above tables seem to justify the following conclusions.

Atrophy is associated with weight loss of roughly the same magnitude following denervation and tenotomy and amounting to some 30 per cent over a period of 20 days. Disuse is attended by a weight loss of some 20 per cent over the same period.

Atrophy of all kinds is associated with a reduction of the total nitrogen content of the tissues, and this decrease is probably dependent on the fatty infiltration of the tissues which simultaneously takes place.

In addition all forms of atrophy give rise to a diminution of the myofibril protein fraction and also to an increase of the stroma protein fraction, the latter being most marked following denervation atrophy. The non protein nitrogen shows only minor changes.

The sarcoplasm proteins appear to change in different directions depending on the type of atrophy. The amount of these proteins is lower following denervation atrophy. After disuse atrophy, on the other hand, their absolute amount remains practically unchanged, but this corresponds to a relative increase. The tenotomized animals take an intermediate position in this respect.

These changes accordingly imply that denervation atrophy gives rise to a decrease of the main protein components while disuse atrophy causes a selective reduction of the contractile proteins.

Finally it may be mentioned that normal and atrophic muscles exhibit no marked differences as regards the relative dry weight. Other deviations of the mutual relationships between the several nitrogen components will be found in Tables 14 to 16.

Discussion. The experiments made revealed that the effects of denervation atrophy and disuse atrophy are not the same.

In my view this difference may be explained as follows. Neurotomy produces complete immobilization of the muscles so that they no longer can be made to contract. This results in a slow wasting away of the muscular tissue as a whole. Disuse, on the other hand, restricts the activity of the muscles but does not put them out of action. The result is, as indicated in Chapter X, that adaptation takes place: the muscles adapt themselves to their altered working conditions, and this is manifested by a selective reduction of their contractile protein content.

GENERAL SUMMARY

The introduction supplies a brief account of the background of the present investigation. The constitution of the muscle cell is described and the functions of the sarcoplasm and myofibrils are explained — while the myofibrils are responsible for the contractility, the sarcoplasm has an important function as storage place for muscle cell nutrients and it also participates in the intermediate metabolism. A measure of the contractile power of the muscle cell can be obtained by estimating its myofibril density. Heretofore only morphological methods have been available for this purpose, and these merely provide a rough approximation of the ratio of sarcoplasm quantity to myofibril quantity. Hence a procedure for quantitative determination of the amounts of these structures present under different circumstances would be a valuable tool, but adequate methods for making such determinations are not described in the extant literature. Accordingly I made it my aim to establish whether it would be feasible to develop a simple procedure for quantitative determinations of the protein fractions associated with the sarcoplasm as well as of those in the myofibrils. If so, I would proceed to apply the method in an analysis of the sarcoplasm protein and myofibril protein contents of muscular tissue under normal and abnormal, in this instance mainly atrophic, conditions.

Chapter I reviews previous studies on muscle proteins. It is described how these proteins have been extracted and their main properties are mentioned, particular emphasis being given to the fundamental physico-chemical dissimilarities exhibited by the sarcoplasm and the myofibril (contractile) proteins. The sarcoplasm proteins are readily soluble, occur in globular form and have low viscosity and low molecular weight. The myofibril proteins are refractory to extraction and have a fibrous nature, high viscosity and high molecular weight.

Chapter II discusses previous analyses of the quantitative protein composition of normal muscular tissue. Comparatively few papers deal with this subject. These investigations are reviewed and criti-

cised, the principal objections being raised against the methods of calculating the protein quantities, the extremely complicated extraction procedures, and the fact that the calculated quantities of stroma proteins have been excessive due to incomplete extraction of the fibre proteins. The chapter also includes a brief account of previous attempts to evaluate the quantitative protein composition of atrophic muscular tissue. The investigations in question involve the use of preparation and extraction procedures that are open to criticism on the same points as those considered in the preceding sentence.

These chapters (I—II) may be regarded as an introduction to the rest of the text which is divided into two parts. The first of these parts (chapter III—VIII) deals with the problem of how quantitative extraction of muscle proteins can be achieved. In it I describe a series of experiments whose results were used as the basis for the method ultimately developed.

Chapter III accounts for the materials and general methods employed in the investigation.

Chapter IV deals with experiments with various techniques for preparation of muscular tissue (1—5). The following experiments were made.

1. After the animal had been killed it was exsanguinated in a very simple manner. It is subsequently shown, with the aid of P^{32}-labelled red blood corpuscles, that only about 1 per cent of the blood remained in the muscular tissues.

2. Various means for subdividing the muscular tissue were tried and mutual comparisons made between the following 7 techniques: a) unsubdivided compact muscle block, b) muscle ground in meat mincer, c) muscle cut to pieces with scissors, d) muscle cut to pieces with scissors and then ground with sand in mortar, e) muscle homogenized in all-glass homogenizer, f) muscle sectioned (into 20 μ sections) with freezing microtome, and g) muscle sectioned with freezing microtome and ultrasonically treated. The method of choice proved to be sectioning with the freezing microtome. No improvement resulted from the ultrasonic treatment.

3. Since it is important to store muscular tissue well chilled (cf. fuller information under 4 below) prior to extraction, I stored the frozen muscular tissue sections in carbon dioxide snow. The sections were also weighed while in the frozen state. As doing this meets

with certain technical difficulties, I have accounted for my weighing procedure in detail and also demonstrated that no carbon dioxide snow adheres to the sections and no water vapour condenses within or on the vessel containing the sections during the weighing process.

4. This experiment was designed to evaluate the effect of muscular tissue storage temperature on the amount of proteins extracted. Muscular tissue was accordingly stored at different temperatures in one series before and in another after sectioning with the freezing microtome. The temperatures selected were +20° and +8° C, that of ice water and that in a deep freezer (−20° C). It appeared that muscular tissue cannot be stored at +20° or +8° C or in ice water without a reduction of the extraction yield. Muscular tissue can be stored in a deep freezer for up to 48 hours without undergoing such changes.

5. The aim of this experiment was to establish whether the so-called Deuticke effect makes itself felt in conjunction with the extraction procedure used henceforth. On test animals under local anaesthesia repeated contractions were induced by unilateral electrical stimulation of the sciatic nerve, whereupon muscular tissue was excised and extracted, the protein yield from the stimulated side being compared with that from the contralateral unstimulated side. No differences were manifest.

Chapter V is concerned with a number of experiments aimed to achieve a maximal nitrogen yield with the use of various extraction procedures (6—12). The following experiments were made.

6. The yields of extraction at different pH levels between 3.6 and 9.7 were compared, the highest yield being obtained in the pH range 6.5 to 9.0.

7. A number of cations (Mg, Ca, K, Na and Li) and anions (Cl, Br and I) were compared with respect to their effect on the amount of proteins extracted. It appeared that the greatest yield was obtained with KI solution as the extractant. When KCl and KI were mixed in different proportions, it was found that the higher the KI concentration in the extractant the greater was the yield.

8. This experiment was intended to clarify the effect of different additives. The actions of adenosine triphosphate, pyrophosphate and a detergent were studied, but the use of these additives did not increase the amount of proteins extracted.

9. The object of this experiment was to fix the best ionic strength of KCl and KI extractants. It was found — as regards both KCl and KI — that the highest protein yield was obtained by extracting with 1.1 M solutions buffered with 0.1 M K phosphate to pH 7.4. The combined ionic strength of such a solution is about 1.3.

10. The next item studied was the influence of the duration of the extraction. It appeared that with 0.03 M K phosphate buffer, pH 7.4, the yield of sarcoplasm proteins reaches maximum in 3 hours. If use is made of 0.6 M KCl, a typical extractant adopted by previous investigators, the extraction must be continued for 10 hours before the yield becomes maximal. With 1.1 M KCl or KI extractants such a yield is attained after as little as 3 hours.

11. The purpose of this experiment was to determine the optimum extractant volume. It was found that increasing yields were obtained with increasing extractant volumes, but also that the incremental yield was negligible with more than 10 aliquots of extractant.

12. This experiment, where extraction was repeated until no more proteins were recovered, revealed that the sarcoplasm proteins were exhaustively extracted by 2 consecutive extractions of 3 hours each with 0.03 M K phosphate buffer, pH 7.4, whilst the myofibril proteins were exhaustively extracted by 3 consecutive extractions for 3, 3 and 2 hours respectively with 1.1 M KI + 0.1 M K phosphate buffer, pH 7.4. In each case measurable quantities of non protein nitrogen were recorded after the first extraction only.

Chapter VI describes the results of analyses of the protein extract obtained. The following experiments (13—16) were made.

13. Trials were made with several methods of separating the muscle proteins obtained into more main components than those considered here, *viz.* sarcoplasm proteins and myofibril proteins. After salting out, paper electrophoresis and ultracentrifugation had been found unsuitable for one reason or another, electrophoresis in the Tiselius apparatus was tried. This technique resulted in separation of the various components in the extract containing sarcoplasm proteins. The extract was free from contractile proteins. The result agreed with previous findings reported in the literature. Electrophoretic separation of the proteins in the extract obtained with 1.1 M KI could not be achieved without prior dialysis to lower ionic strength. However, myosin was then precipitated. Electrophoresis

was nevertheless carried out at ionic strength 0.25 and the results are described.

14. This was a viscosimetric experiment which disclosed that the extract obtained with 0.03 M K phosphate buffer, pH 7.4, was free from actomyosin.

15. Examination of the extracts under the phase contrast microscope disclosed so very few cell fragments that their presence scarcely could cause an artificial increase of the amount of nitrogen extracted.

16. This experiment was made to exclude the eventuality that the extracts contained freely suspended submicroscopic particles in sufficient numbers to artificially increase the amount of nitrogen extracted. The extracts were first analyzed after centrifugation at 1,400 g and then after centrifugation at 25,000 g. This disclosed no differences between the nitrogen contents of the centrifuged extracts; and, as even the smallest granulae sediment at 19,000 g, the extracts cannot have contained measurable amounts of such freely suspended submicroscopic particles.

Chapter VII accounts for the experiments made with the muscular tissue remaining after completed extraction with 1.1 M KI + 0.1 M K phosphate buffer, pH 7.4. The following control experiments were made in order to prove that the extraction had been exhaustive (17—19).

17. Histological examination of slides with stained and unstained tissue residue revealed no muscle cell remnants.

18. Tropomyosin could not be extracted by Bailey's method.

19. Actin could not be extracted by Straub's method. As actin and tropomyosin are considered the most difficult to extract, and as these substances nevertheless had been exhaustively extracted, it was concluded that all other more soluble proteins had been extracted exhaustively.

Chapter VIII summarizes the optimal conditions for quantitative extraction and determination of sarcoplasm and myofibril proteins, against the background of the experiments described in Chapters III through VII. It goes on to define a method for calculation of stroma proteins. Values are given for the protein fractions in a typical analysis and these are related to the composition of muscular tissue.

In the second part of the book (Chapter IX—XI) I narrate the results obtained by practical application of the method developed for

muscle protein extraction. Analyses were made both on normal muscles of various types and on atrophic skeletal muscle.

Chapter IX specifies materials and general methods.

Chapter X outlines the results of analyses of normal muscles from rabbits and cattle. Analyses were made on striated muscle from different regions. It appeared from the results that the protein composition varies considerably from one type of muscular tissue to another, and I concluded that this variability is a manifestation of functional adaption — the more work a muscle has to do, the greater its proportion of contractile proteins seems to be, and vice versa.

Chapter XI deals with a number of experiments where atrophy was induced experimentally in rabbits by means of neurotomy, tenotomy and immobilization with plaster casts. Twenty days after the operation or application of the cast, the degree of denervation and tenotomy atrophy was some 30 per cent and that of disuse atrophy some 20 per cent. In all the experiments it was found at the end of the observation period that the total nitrogen content of the muscular tissue had diminished while the water content was essentially unchanged. Analyses of the separate nitrogen components showed that the myofibril protein content had decreased and the stroma protein content had increased in all cases. The non protein nitrogen content showed minor changes only. The sarcoplasm protein content, lastly, increased or decreased depending upon the type of atrophy. While it decreased after denervation, it remained practically unchanged after disuse, *i. e.* sarcoplasm proteins comprised a larger percentage of the atrophic tissue. The effect of tenotomy atrophy was intermediate in this respect. I interpreted these differences as follows. In a denervated muscle general degeneration takes place, both myofibrils and sarcoplasm are involved, while in a cast-immobilized muscle the contractile elements are selectively reduced. This is probably associated with the fact that whereas denervation makes the muscle completely inactive, the plaster cast merely restricts muscular activity. In this connection, therefore, the selective reduction of the myofibril protein content should probably be regarded as a manifestation of an adaptation process in the muscle cell.

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